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(54) Title: INHIBITION OF P70 S6 KINASE (57) Abstract A method of screening for an antiproliferative or immunosuppressive agent, which method includes the steps of (1) contacting a eukaryotic cell with a candidate antiproliferative or immunosuppressive composition; and (2) determining the level of activity of a serine/threonine kinase or a serine/threonine phosphatase in the p70 S6 kinase cascade of said cell in the presence of the candidate composition, wherein a level of said activity that results in a lower p70 S6 kinase activity in the presence of the composition than in the absence of the composition is an indication that the composition is antiproliferative or immunosuppressive agent; and methods of treatment using such compositions.		

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INHIBITION OF P70 S6 KINASE

The invention was made in the course of work funded in part by a grant from the U.S. government, which has certain rights in the invention. The field of the invention is antiproliferative or immunosuppressive agents.

Background of the Invention

A conserved response of many eukaryotic cell types to mitogenic signals is the phosphorylation of multiple serine residues on the 40S ribosomal subunit protein S6 (Erikson, J. Biol. Chem. 266:6007-6010, 1991). This phosphorylation increases the efficiency of protein synthesis, which appears to be required in several steps of cell cycle progression (Erikson, *supra*). Recently, two families of S6 kinases have been characterized at the enzymatic and molecular levels: the 85-90 kDa (rsk) S6 kinase family (Jones et al., Proc. Natl. Acad. Sci. USA 85:3377-3381, 1988), referred to herein as p90 S6 kinase, and the 70 kDa S6 kinase family (Banerjee et al., Proc. Natl. Acad. Sci. USA 87:8550-8554, 1990; Kozma et al., Proc. Natl. Acad. Sci. USA 87:7365-7369, 1990), referred to herein as p70 S6 kinase. The activity of both types of kinases is regulated by serine/threonine phosphorylation (Erikson, *supra*), and both are serine/threonine kinases themselves.

Eukaryotic cells contain many different kinases as part of various cascades that transmute signals from cell-surface receptors to effector molecules within the cell. In some cases the cell-surface receptor is itself a kinase that is activated upon binding its ligand; in other cases, the receptor is associated with a separate protein that acquires kinase activity when the receptor

sequentially phosphorylating (if it is a kinase) or
10 dephosphorylating (if it is a phosphatase) certain
critical residues in the next member of the cascade,
thereby activating or deactivating such next member, as
the case may be.

In T cells expressing the interleukin-2 (IL-2)
15 receptor, binding of IL-2 to this receptor triggers a
response culminating in proliferation of the T cell
(Smith, Ann. Rev. Immunol. 2:319-333, 1984). Evans et
al. observed that one aspect of the IL-2 generated
response is an increase in S6 phosphorylation and a
20 concomitant increase in the rate of protein synthesis (J.
Biol. Chem. 262:4624-4630, 1987). Similarly, the multi-
faceted response of insulin-dependent H4 hepatoma cells
to stimulation by insulin includes an increase in the
level of S6 phosphorylation, apparently attributable at
25 least in part to increased activity of the S6 kinases.

Rapamycin and FK506 are macrolide antibiotics
which are potent immunosuppressive agents. Although the
two compounds share certain structural features (see Fig.
9) and are capable of binding to the same family of
30 cellular proteins (termed FK506-binding proteins, or
FKBPs) to form a biologically active complex, their
mechanisms of immunosuppression differ significantly.
The FK506/FKBP complex inhibits a very early step in
antigen-induced T cell activation, preventing
35 proliferation of activated T cells by blocking the

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induction of cytokine gene transcription. Liu et al. (Cell 66:807-815, 1991) have recently shown that this occurs through the binding of the FK506/FKBP complex to calcineurin, a calcium-dependent phosphatase thought to play a role in T cell signal transduction, thereby blocking the phosphatase activity of this enzyme and short-circuiting signal transduction along this pathway. In contrast, the rapamycin/FKBP complex apparently does not bind to or affect the activity of calcineurin, and acts by blocking a later step in antigen-induced T cell activation, one that occurs subsequent to binding of IL-2 to its receptor (i.e., at a point after the initial induction of cytokine gene transcription). The entity directly targeted by the rapamycin/FKBP complex has not been identified, although it has been shown not to be the IL-2 receptor itself.

Summary of the Invention

As disclosed herein, it has now been shown that the addition of rapamycin to an IL-2-stimulated T cell line results in the inhibition of p70 S6 kinase, which kinase may constitute the direct target of the rapamycin effector complex, or may be downstream of the actual target. Furthermore, experiments are herein disclosed which demonstrate that rapamycin will inhibit both basal and insulin-stimulated proliferation and p70 S6 kinase activity in an insulin-dependent hepatoma cell line, an observation that has broad implications with respect to novel medical applications for rapamycin. The information linking p70 S6 kinase inhibition to the immunosuppressive and newly discovered general antiproliferative effects of rapamycin may be utilized to design an assay for screening for immunosuppressive and/or antiproliferative agents which act by the same or a related mechanism to that of rapamycin: i.e., which

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ultimately result in a decrease in p70 S6 kinase activity in the treated cell. Such an assay will pick up not only those compounds or complexes which act on the same enzyme as that targeted by the rapamycin/FKBP complex, but also those which act upstream or downstream of that particular enzyme in the p70 S6 cascade. The screened compounds or complexes (generally referred to as compositions) which result in decreased p70 S6 kinase activity may act to inhibit a kinase (e.g., p70 S6 kinase itself, or a kinase upstream of p70 S6 kinase), or may activate a phosphatase (e.g., one which specifically dephosphorylates and thus inactivates p70 S6 kinase, or an enzyme upstream of p70 S6 kinase). The screen will also identify those compositions which activate a kinase or inhibit a phosphatase upstream of p70 S6 kinase, provided that this activation or inhibition ultimately results in a decrease in p70 S6 kinase activity.

The screening method of the invention includes the steps of (1) contacting a eukaryotic cell (e.g., a mammalian cell such as a human cell) with a candidate antiproliferative or immunosuppressive composition; and (2) determining the level of activity of a serine/threonine kinase or a serine/threonine phosphatase in the p70 S6 kinase cascade of said cell in the presence of the candidate composition, wherein a level of said activity that results in a lower p70 S6 kinase activity in the presence of the composition than in the absence of the composition is an indication that the composition is an antiproliferative or immunosuppressive agent. The serine/threonine kinase may be p70 S6 kinase itself, or a kinase which phosphorylates and thereby activates p70 S6 kinase *in vivo*, or a kinase which acts upstream of that point. The serine/threonine phosphatase may be a phosphatase which dephosphorylates and thereby inactivates p70 S6 kinase *in vivo*, or a phosphatase which

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acts upstream of that point. In preferred embodiments, step 2 may be readily accomplished by simply measuring the level of p70 S6 kinase activity, which is directly or inversely related to the activities of each of the

5 kinases and phosphatases upstream of p70 S6 kinase in the p70 S6 kinase cascade. In order to maximize the window of response in this assay, the cell may optionally be exposed to an appropriate mitogen (preferably a growth factor) before or during step 1. For example, where the

10 cell is a hematopoietic cell, a cytokine such as interleukin 1 (IL-1), IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, erythropoietin (EPO), Steel factor (stem cell factor); granulocyte colony stimulating factor (G-CSF), macrophage colony stimulating factor (M-

15 CSF), or granulocyte/macrophage colony stimulating factor (GM-CSF) may be used to trigger proliferation signals; preferably, the cell is a lymphocyte such as a T cell or B cell, and the mitogen of choice is a lymphokine such as one of the interleukins.

20 Alternatively, candidate antiproliferative or immunosuppressive agents (including, for example, analogs of rapamycin and analogs of the rapamycin/FKBP complex) may be screened *in vitro* in a method including the following steps:

25 (1) combining (a) a sample containing p70 S6 kinase, (b) a substrate for the kinase, and (c) a candidate antiproliferative or immunosuppressive composition; and

(2) determining whether the candidate composition

30 inhibits the biological activity of the kinase, such inhibition being an indication that the candidate composition is an antiproliferative or immunosuppressive agent. Alternatively, the *in vitro* assay may employ p70 S6 kinase as the substrate, and an enzyme just upstream

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of p70 S6 kinase as the effector. This method would include the following steps:

(1) providing a sample containing p70 S6 kinase and either (i) a serine/threonine kinase capable of phosphorylating and activating p70 S6 kinase, or (ii) a serine/threonine phosphatase capable of dephosphorylating and deactivating p70 S6 kinase;

(2) contacting the sample with a candidate antiproliferative or immunosuppressant composition; and

(3) determining whether the amount of phosphorylation of the p70 S6 kinase in the presence of the candidate composition is lower than the amount of phosphorylation of p70 S6 kinase in the absence of the candidate composition, such a lower amount of phosphorylation being an indication that the candidate composition is an antiproliferative or immunosuppressive agent.

The invention also includes methods of inhibiting the proliferation or immune response of a cell (e.g., a hematopoietic cell such as a T cell, stimulated by a cytokine such as IL-2) of an animal (preferably a mammal such as a human), by introducing into the animal a composition that interrupts the p70 S6 kinase cascade via a mechanism such as (i) directly inhibiting the activity of the p70 S6 kinase of the cell, or (ii) directly inhibiting the activity of a kinase which phosphorylates *in vivo* a serine or threonine residue on p70 S6 kinase, or (iii) directly increasing the activity of a phosphatase which dephosphorylates *in vivo* a serine or threonine residue on p70 S6 kinase. By "directly" is meant that the altered activity of the given kinase or phosphatase results from the interaction between the composition (or a complex containing the composition) and the given kinase or phosphatase, and not between the composition and an enzyme upstream to the given kinase or

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phosphatase. Although it is not known which, if any, of these possible mechanisms is the mechanism of rapamycin/FKBP, the experimental results obtained with rapamycin indicate that a composition which functioned by
5 any one of these mechanisms would produce the desired result.

Also within the invention is a method of inhibiting cellular proliferation in response to a mitogen other than IL-2, which method includes the steps
10 of (1) providing a cell which proliferates in response to the mitogen; and (2) treating the cell with a composition (which may include rapamycin or an analog of rapamycin) that modulates the activity of a serine/threonine kinase (such a p70 S6 kinase or a kinase which activates p70 S6
15 kinase) or a serine/threonine phosphatase (such one which dephosphorylates, and thereby deactivates, p70 S6 kinase) in the p70 S6 kinase cascade of the cell, thereby resulting in a decrease in the activity of p70 S6 kinase in the cell. This method may be used to treat an animal
20 having a condition characterized by proliferation of a cell in response to the given mitogen. The term mitogen is meant to include any entity which, when contacted with a cell, stimulates the cell to proliferate at a rate higher than the rate in its absence. Examples of such
25 mitogens include lectins and growth factors (defined as naturally-occurring proteins or glycoproteins which acts as endogenous mitogens *in vivo*) such as IL-1, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, Steel factor, G-CSF, M-CSF, GM-CSF, EPO, epidermal growth factor (EGF),
30 fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), and insulin.

Also within the invention is an analog of rapamycin or of the rapamycin/FKBP complex, which analog, when introduced into a cell, modulates the activity of a
35 serine/threonine kinase or serine/threonine phosphatase

preparation of such analogs is well within the abilities of one of ordinary skill in the art of synthetic organic chemistry. Given the simple and rapid assays provided herein for determining whether a given analog functions
15 in the same manner as rapamycin, the rapid screening of large numbers of such analogs is now feasible.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

20 Detailed Description

The drawings are first described.

Drawings

Fig. 1 is a set of SDS-PAGE gels illustrating the effect of IL-2 and PMA on S6 kinase activity, MAP kinase
25 activity, and tyrosine phosphorylation in CTLL-20 cells. Cells were treated with IL-2 or PMA for the indicated times. Lysates were analyzed for (A) total S6 kinase activity, (B) S6 kinase activity due to p90 S6 kinase, (C) MAP kinase activity, or (D) induction of tyrosine
30 phosphorylation. For the *in vitro* kinase assays (A-C), the positions of the corresponding substrates are indicated on the left. For the antiphosphotyrosine immunoblot (D), the positions of molecular weight markers (in kDa) are indicated on the left.

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Fig. 2 is a graph and a set of immunoblot autoradiograms illustrating the chromatographic resolution of p70 and p90 S6 kinases. IL-2-deprived cells were either left untreated or treated with rapamycin for 60 min, and either left unstimulated or stimulated with IL-2 for an additional period of 60 min. Cell lysates were applied to a Mono Q anion-exchange column and eluted with a linear gradient of NaCl from 0.0 to 0.5 M, and fractions were collected and analyzed.

10 (A) S6 kinase activity. Open circles: untreated, unstimulated. Closed triangles: untreated, stimulated with IL-2. Closed circles: treated with rapamycin, stimulated with IL-2.

(B) Immunoblotting with p70 S6 kinase-specific antiserum.

15 (C) Immunoblotting with p90 S6 kinase-specific antiserum. Treatments and stimulations are indicated on the left, fraction numbers are indicated at the bottom, and the positions of molecular weight markers (in kDa) are indicated on the right. The positions of p70 and p90 S6

20 kinases are indicated with arrows on the right. The 90 kDa proteins detected by the anti-p70 S6 kinase antiserum (fractions 14-15) correspond to the recently described high molecular weight forms of the p70 S6 kinases (Grove et al., Mol. Cell. Biol. 11:5541-5550, 1991). Note that

25 the protein bands migrating at approximately 100-110 kDa which are recognized by the p70 S6 kinase-specific antiserum (fractions 9-10 and 19-21) elute in fractions devoid of detectable S6 kinase activity.

Fig. 3 is a set of SDS-PAGE gels illustrating the

30 effects of rapamycin and FK506 on S6 kinase activity, MAP kinase activity, and tyrosine phosphorylation in CTLL-20 cells. Cells were preincubated with rapamycin or FK506 as indicated and either left untreated for 5 min (lanes 1-3), treated with IL-2 for 5 min (lanes 4-6) or 60 min

35 (lanes 7-9), or treated with PMA for 5 min (lanes 10-12).

glycerophosphate/0.1% Triton X-100/2 mM leupeptin/2mM
pepstatin/0.2 mM PMSF); balanced for protein; and assayed
for S6 kinase activity according to Nemenoff (Arch.

20 Biochem. Biophys. 245:196-203, 1986).

Fig. 4B is an SDS-PAGE gel illustrating the in
vivo incorporation of $^{32}\text{P}_i$ into ribosomal protein S6. H4
hepatoma cells (24-hr serum-starved) were washed with
phosphate-free, serum-free DMEM, then incubated with 0.5
25 mCi $^{32}\text{P}_i$ /plate in DMEM for 1 hr. Rapamycin was then added
at the indicated final concentrations (in quadruplicate).
After 1 hr of rapamycin incubation, one half of the
plates were incubated for 30 min with 150 mU/ml insulin
(10^{-6} M), and the other half left untreated. Cells were
30 then harvested in 0.5 ml extraction buffer (10 mM KP_i , pH
6.5/1 mM EDTA/5mM EGTA/10 mM MgCl_2 /2 mM DTT/1 mM VO_4 /
50 mM β -glycerophosphate/2 mM leupeptin/2 mM pepstatin/
10 U/ml aprotinin/0.2 mM PMSF). Cells were broken in a
dounce homogenizer and centrifuged 10 min at 1000 x g.
35 Supernatants were then balanced for protein (BioRad), and

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a an aliquot was centrifuged. The pellet was resuspended in 200 ml of extraction buffer including 0.1% Triton X-100, and then treated with SDS sample buffer and run on a 15% polyacrylamide gel (sample balanced for BioRad protein).

Fig. 5 is a set of graphs depicting the results of Mono Q chromatography of H4 hepatoma extracts. H4 hepatoma cells, serum-starved as in Fig. 4, were treated with 10^{-6} M insulin for 30 min (left panels), or 10^{-6} M insulin (30 min) preceded by a 1 hr incubation with 20 nM rapamycin (right panels). Cells were extracted in homogenization buffer and centrifuged as in Fig. 4. Extracts were diluted 3-fold in chromatography buffer (50 mM β -glycerophosphate, pH 7.2/1mM DTT/1mM EGTA/0.1mM vanadate) and applied to a Mono Q HR (5/5) column equilibrated with chromatography buffer. The column was eluted with a 90 ml gradient (0-0.4 M NaCl) in chromatography buffer with collection of 1 ml fractions. Individual fractions were assayed for kinase activities using 40S ribosomes and SKAIPS peptide. Fractions were also assayed for total protein and conductivity.

Fig. 6 is a set of SDS-PAGE gels illustrating the time course of insulin-stimulated S6 kinase activity in the presence and absence of rapamycin. Serum-starved H4 hepatoma cells were incubated in the presence (right) or the absence (left) of 20 nM rapamycin for 1 hr at 37°C, 5% CO₂, after which cells were harvested and extracts prepared. Top panel: Extracts were assayed for total S6 kinase activity using 40S ribosomes as substrates for phosphorylation. Middle panel: Extracts were immunoprecipitated with an affinity-purified polyclonal p70 S6 kinase antibody (1 μ g/ tube) in the presence of 10 μ l protein A Sepharose beads. After a 3 hr incubation at 5°C, immunoprecipitated material was washed 3X in homogenization buffer and 0.25 M NaCl + 0.1% Triton X-

for S6 kinase activity using
5 substrate. Lower panel: Extracts were immunoprecipitated with an affinity-purified polyclonal p90 S6 kinase antibody (1 μ g per tube) as in the middle panel, followed by a determination of S6 kinase activity.

Fig. 7A is a pair of graphs comparing the effects
10 of rapamycin on p70 S6 kinase, p90 S6 kinase, and erk1/MBP kinase. COS cells were transfected with expression constructs encoding the rat p70 S6 kinase (Grove et al., Mol. Cell. Biol. 11:5541-5550, 1991), the rat p90 S6 kinase or the rat p44 erk1/MBP kinase, all of
15 which had been tagged at the amino terminus with a nonapeptide epitope from influenza hemagglutinin (Field et al., Mol. Cell. Biol. 8:2159-2165, 1988). Two days following transfection, the cells were treated with the indicated concentrations of rapamycin for 60 min and with
20 100 nM phorbol 12-myristate 13-acetate (PMA) for the final 15 min. Cells were lysed with homogenization buffer [as in Fig. 4a, but containing 0.1% (w/v) Triton X-100], and the extract was clarified by centrifugation at 100,000 x g. Recombinant protein was
25 immunoprecipitated with the anti-epitope antibody 12CA5 (immobilized on Sepharose beads) for 3 h at 4°C; pellets were washed (3 x 1 ml) in assay buffer [homogenization buffer without the $MgCl_2$ and with 10% (w/v) glycerol]. S6 kinase activity was determined as above and p44
30 erk1/MBP kinase was assayed similarly, using 0.5 mg/ml myelin basic protein (MBP) as substrate. Results are expressed as the mean pmol of substrate labeled during the 15 min assay; error bars indicate standard deviation of replicate determinations (upper panel, n=5; lower

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panel, n=6). The insert shows a Western blot of the recombinant p70 protein detected with the 12CA5 monoclonal antibody and visualized by chemiluminescence (ECL, Amersham), demonstrating that recovery in each of the immunoprecipitants was equivalent.

Fig. 7B is a bar graph which presents data indicating that rapamycin but not FK506 inhibits recombinant p70 S6 kinase. COS cells were transfected with vector or a p70 S6 kinase expression construct, as indicated; treated with 100 nM PMA and with 2 nM rapamycin or 200 nM FK506 (as indicated); and extracted, immunoprecipitated, and assayed as in 7A. The insert shows a Western blot of the recombinant p70 protein detected with the 12CA5 monoclonal antibody and visualized by chemiluminescence, demonstrating that recovery in each of the immunoprecipitants was equivalent.

Fig. 7C is a graph, an autoradiogram of an SDS-PAGE gel, and a Western blot illustrating the rapamycin dose response of p70 S6 kinase with respect to autophosphorylation of p70 S6 kinase and phosphorylation of 40S ribosomal subunits. COS cells were transfected, treated with rapamycin, extracted, and immunoprecipitated as in 7A. *Upper panel*: 40S kinase activity was determined from replicate sampling of duplicate immunoprecipitations (mean and standard deviation, n=6). *Middle panel*: Autophosphorylation was determined from kinase assays lacking substrate using aliquots of the immunoprecipitations from cells transfected with vector or recombinant p70 S6 kinase (as indicated) and treated as indicated with no addition or with rapamycin at the following concentrations: 0.03 nM, 0.1 nM, 0.3 nM, 1 nM, or 3 nM. *Lower panel*: Western blot of samples in the same order as in the middle panel. Recombinant p70 protein was detected with the 12CA5 monoclonal antibody

presence of rapamycin as
Proliferation was assessed by the incorporation of [³H]-
15 thymidine in a 16 hr pulse following a 32 hr incubation.
This experiment is representative of four similar
experiments.

Fig. 9 is an illustration of the chemical and 3-
dimensional structures of FK506, rapamycin, and 506BD
20 (Bierer et al., Science 250:556-559).

Example 1: Inhibition of IL2 stimulation of p70 86
kinase

MATERIALS AND METHODS

Cell culture and stimulation of cells. The IL-2-
25 dependent murine cell line CTLL-20 (American Type Culture
Collection, Rockville, MD) was cultured in RPMI-10%FCS
medium as described (Calvo et al., Eur. J. Immunol.
22:457-462, 1992, herein incorporated by reference),
containing human recombinant IL-2 (12.5-25 units/ml,
30 kindly donated by Hoffmann-LaRoche, Inc.). Cells were
recovered by centrifugation, washed 3 times with RPMI
1640; resuspended in RPMI-10%FCS at $1-5 \times 10^6$ cells/ml
and incubated for 3 h at 37° C. Cells were left
untreated or treated with 100 nM rapamycin, 100 nM FK506

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or equivalent ethanol diluent (final concentration 0.1%) for the last hour and during the subsequent stimulation period. Aliquots of $4-10 \times 10^6$ CTLL-20 cells were either left untreated or incubated in the presence of human
5 recombinant IL-2 (25 units/ml) or phorbol-12-myristate-13 acetate (PMA, 50 ng/ml, Sigma) for the indicated times at 37°C, and then divided into two aliquots. *In vitro* kinase assays. *In vitro* kinase assays were performed essentially as described (Calvo et al., *supra*). 1-2 x
10 10^6 cells were spun after stimulation and lysed in 0.2 ml of 10 mM potassium phosphate, pH 7.05, 1 mM EDTA, 0.5% Triton X-100, 5 mM EGTA, 10 mM $MgCl_2$, 50 mM β -glycerophosphate, 1 mM sodium vanadate, 2 mM DTT, 40 μ g/ml PMSF, 10 μ g/ml leupeptin, and 1 μ g/ml pepstatin.
15 After 30 min on ice, nuclei were pelleted by a 5-min microfuge centrifugation, and supernatants were used for kinase assays.

For the detection of total S6 kinase activity, direct S6 phosphorylation assays were performed with 5 μ l
20 of supernatant in a total volume of 30 μ l containing 20 mM Tris-HCl pH 7.25, 10 mM $MgCl_2$, BSA 10-100 μ g/ml, 50 μ M ATP with 5 μ Ci [γ - 32 P]ATP, and 40S ribosomal subunits as substrate (0.1-0.25 mg/ml) prepared from *Xenopus laevis* as described (Erikson et al., J. Biol. Chem. 261:350-355,
25 1986). For S6 kinase assays specific for p90 S6 kinase, 100 μ l of supernatant were incubated with 5 μ l of the specific rabbit antiserum 125 (Sweet et al., Mol. Cell. Biol. 10:2787-2792, 1990), and immunocomplexes were adsorbed to *Staphylococcus aureus*, washed as previously
30 described (Vik et al., Proc. Natl. Acad. Sci USA 87:2685-2689, 1990), and subjected to S6 phosphorylation assays as described above.

To assay for MAP kinase activity, 5 μ l of supernatant were used in a total reaction volume of 30 μ l
35 containing 20 mM Tris-HCl pH 7.25, 10 mM $MgCl_2$, 100 μ M

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ATP with 5 μ Ci [γ - 32 P]ATP, and \sim 1 μ g of unactivated Xenopus p90 S6 kinase (referred to as rsk) obtained as described (Vik et al., *supra*). In all cases kinase reactions were carried out at 30°C for 15 min and
5 analyzed as previously described (Calvo et al., *supra*).

Antiphosphotyrosine immunoblotting analysis. 3-8 $\times 10^6$ cells were washed, lysed, and centrifuged as described (Calvo et al., *supra*). The protein concentration in the supernatants was determined by a
10 colorimetric method (Bradford, Anal. Biochem. 72:248-254, 1976). Equivalent protein amounts of each supernatant were resolved by reducing 8-10% SDS-PAGE and analyzed by immunoblotting with the antiphosphotyrosine mAb 4G10 and anti-mouse IgG alkaline phosphatase-conjugated antibody
15 (Promega) as described (Calvo et al., *supra*).

Ion-exchange chromatography and anti-p70/p90 S6 kinase immunoblotting analysis. 1.4×10^8 cells were lysed in homogenization buffer (5 mM EGTA, 1 mM EDTA, 10 mM MgCl_2 , 50 mM β -glycerophosphate, 2 mM DTT, 0.2% Triton
20 X-100, 10% glycerol, 1 mM PMSF, 10 μ g/ml leupeptin, and 10 μ g/ml pepstatin) and centrifuged at 100,000 $\times g$ for 30 min at 4°C. Supernatants, normalized for total protein content, were applied to a Mono Q (Pharmacia) anion-exchange chromatography column and bound proteins
25 were eluted in a 18-ml gradient of 0-500 mM NaCl (Grove et al., Mol. Cell. Biol. 11:5541-5550, 1991). Aliquots (5 μ l) of each 0.75 ml fraction were assayed for S6 kinase activity as described above. Aliquots (16 μ l) of each fraction were resolved by reducing 8% SDS-PAGE and
30 analyzed by immunoblotting with the p90-specific rabbit antiserum 125 as described (Vik et al., *supra*), and with the 70-kDa S6 kinase specific rabbit antiserum (anti-rat S6 kinase, UBI), as recommended by the manufacturer, using an anti-mouse IgG alkaline phosphatase-conjugated
35 antibody..

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RESULTS

A. IL-2 stimulates S6 kinases of the p70 family in CTLL-20 cells.

The IL-2-dependent murine T cell line CTLL-20 proliferates in response to IL-2 stimulation. CTLL-20 cells were allowed to reach plateau phase of growth, extensively washed, and starved of IL-2 for 3h. IL-2 deprivation for this period of time did not affect cell viability or subsequent IL-2-sustained proliferation (data not shown). IL-2-deprived CTLL-20 cells were stimulated with either recombinant IL-2 or PMA, and total S6 kinase activity was measured in cell lysates by phosphorylation of 40S ribosomal subunits in vitro (Fig. 1A). IL-2 binding resulted in a time-dependent stimulation of S6 kinase activity, which was detectable at 5 min after addition of IL-2 and increased cumulatively until at least 120 min after IL-2 addition. Incubation with PMA also resulted in a marked stimulation of S6 kinase activity which increased over time compared to untreated cells.

To analyze the contribution of the p90 S6 kinase to this IL-2-stimulated increase of activity, aliquots of the cell lysates were immunoprecipitated with an antibody specific for p90 S6 kinases (Sweet et al., *supra*) and S6 kinase activity was measured in the immunocomplexes (Fig. 1B). PMA treatment was found to induce a marked increase in p90 S6 phosphorylating activity measured in the anti-p90 immunocomplex assay. Measured PMA-stimulated p90 S6 kinase activity increased dramatically from time zero to 5 min, and then decreased minimally from 5 to 60 min. In contrast to the results following PMA stimulation, no time-dependent IL-2-induced increase in S6 phosphorylating activity was detected in anti-p90 S6 kinase-immunoprecipitates from cells stimulated with IL-2. These observations suggest that although PMA

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treatment results in the activation of the p90 S6 kinase in these cells, IL-2 treatment does not. Therefore, the stimulation of S6 kinase activity observed following IL-2 receptor occupancy appears to be the result of activation of members of the p70 S6 kinase family.

MAP kinase has been reported to activate the p90 S6 kinase in vitro (Sturgill et al., Nature 334:715-718, 1988). To determine if the lack of activation of p90 S6 kinase by IL-2 correlated with a lack of activation of MAP kinase, MAP kinase assays were performed using p90 S6 kinase as substrate with lysates from either untreated cells or cells incubated with IL-2 or PMA. MAP kinase activity from lysates of IL-2-stimulated cells was similar to or slightly increased compared to untreated cells (Fig. 1C and data not shown). In contrast, PMA-treated cells exhibited a dramatic increase in MAP kinase activity (Fig. 1C). These results demonstrate that MAP kinase in CTLL-20 cells, although strongly activated by PMA treatment, is minimally affected by IL-2 stimulation.

IL-2 stimulation induced the appearance of tyrosine phosphorylated proteins in a time-dependent manner (Fig. 1D). A prominent band of ~100 kDa was observed, as well as a much fainter band of ~60 kDa. In order to be maximally active, MAP kinase requires phosphorylation both on tyrosine and serine/threonine residues (Anderson et al., Nature 343:715-718, 1990). Tyrosine-phosphorylated MAP kinase can be detected by antiphosphotyrosine immunoblotting in cells stimulated by a variety of external signals, including T cell receptor-CD3 (TCR-CD3) stimulation of T cells (Calvo et al., *supra*; Hanekom et al., Biochem. J. 262:449-455, 1989). The presence of tyrosine-phosphorylated 42 kDa protein (the M_r of MAP kinase) in IL-2-stimulated cell lysates varied from undetectable to barely detectable (Fig. 1D and data not shown). This result correlates with the

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lack of a prominent stimulation of MAP kinase activity by IL-2. In contrast, incubation with PMA induced a very prominent tyrosine-phosphorylated 42 kDa band (Fig. 1D) in all experiments performed.

5 To obtain direct evidence that the IL-2-stimulated S6 kinase activity corresponded to S6 kinases of the p70 S6 kinase family, cell lysates were fractionated by Mono Q anion-exchange chromatography which resolves two peaks of S6 kinase activity: p90 S6 kinases elute earlier than
10 p70 S6 kinases in the salt gradient (Grove et al., *supra*). Compared to unstimulated cells, IL-2-stimulated cell lysates contained an increased S6 kinase activity peak which eluted in a position corresponding to p70 kinases (Fig. 2A). To verify that p70 and p90 S6 kinases
15 had been effectively separated, we tested for the presence of these kinases in the different fractions by immunoblotting of SDS-PAGE-resolved aliquots with specific antisera. Anti-p90 S6 kinase immunoreactive proteins concentrate in fractions 5-11 (Fig. 2B), while
20 anti-p70 S6 kinase immunoreactive proteins concentrate in fractions 14-17 (Fig. 2C), which correspond to the peak of IL-2-stimulated S6 kinase activity. Thus, both by chromatographic behavior and immunoreactivity, IL-2 stimulates an increase in S6 kinase activity of the p70
25 family.

B. Inhibition of p70 S6 kinase activity by rapamycin.

Rapamycin is a macrolide antibiotic with immunosuppressive activity. Structurally homologous to
30 FK506, rapamycin has been shown to bind to a family of intracellular proteins termed FK506 binding proteins, or FKBP. While the FK506/FKBP complex has been shown to bind to and inhibit the activity of the serine/threonine phosphatase known as calcineurin (Liu et al., Cell,
35 66:807-815, 1991), the target of action of rapamycin is

1). Rapamycin slightly inhibited the marked stimulation of total S6 kinase activity by PMA in these cells (Fig. 3A, lanes 10 vs 12).

When lysates from rapamycin-treated, IL-2-
15 stimulated cells were fractionated by Mono Q chromatography, no S6 kinase activity could be detected in fractions corresponding to the p70 S6 kinases (Fig. 2A). Since immunoblotting analysis demonstrated that rapamycin-treated, IL-2-stimulated cell lysates contained
20 similar amounts of immunoreactive p70 S6 kinases compared to untreated, IL-2-stimulated (Fig. 2B) or control (data not shown) cell lysates, downregulation of p70 S6 kinases by rapamycin does not explain the lack of S6 kinase activity. Thus, rapamycin appears to inhibit the
25 activation of p70 S6 kinases.

The inhibitory action of rapamycin on the IL-2-stimulated S6 kinase activity was not due to a general effect on the activation of serine/threonine kinases, since rapamycin treatment did not inhibit the PMA-induced
30 stimulation of either p90 S6 kinase activity (Fig. 3B, lane 10 vs 12) or MAP kinase activity (Fig. 3C, lane 10 vs 12). Thus, the modest inhibition by rapamycin of PMA-increased total S6 kinase activity probably reflects the inhibition of the p70 S6 kinase component of the PMA-
35 stimulated S6 kinase activity. Rapamycin did not inhibit

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the IL-2-induced tyrosine phosphorylation of a number of substrates (e.g., the ~100 kDa band, Fig. 3D, lanes 6 and 9 vs 4 and 7). The target of action of rapamycin thus appears to lie between the early activation of tyrosine
5 kinases and the activation of members of the p70 S6 kinase family, which require serine/threonine phosphorylation for their activation.

Example 2: Inhibition of Insulin/Mitogen-Activated p70 S6 Kinase

10 Insulin treatment of serum-starved H4 rat hepatoma cells results in the activation of S6 protein kinases; assays of whole extracts show a progressive increase in total S6 kinase to a plateau at 10 min that is sustained thereafter for at least 1 hr (Fig. 4). Incubation of H4
15 cells with rapamycin for 1 hr prior to insulin addition leads to a dose-dependent (Fig. 4A) and parallel inhibition of basal and insulin-stimulated S6 kinase activities that is essentially complete at 10 nM rapamycin. FK506, a structural analog of rapamycin, and
20 cyclosporin A, a structurally unrelated agent that nevertheless shares a similar biological spectrum of action of FK506, can each also inhibit slightly the insulin-stimulated p70 S6 kinase activity, but with a potency <1% that of rapamycin (data not shown). This
25 inhibition of total extract S6 kinase reflects an inhibition of the p70 S6 kinase, as illustrated by the loss, following treatment of the cells with rapamycin, of (i) p70 S6 kinase activity in immunoprecipitates formed with an anti-p70 peptide antibody (Fig. 4B), and (ii) the
30 characteristic peak of Mono Q activity (see below). The p90 S6 kinase contributes less than 5% of the total extract 40S S6 kinase activity (Fig. 5). Nevertheless, when examined selectively by immunoprecipitation with an anti-p90 peptide antibody, the p90 S6 kinase can be seen

10 tyrosine phosphorylation substrate termed IRS-1. Antiphosphotyrosine immunoblots of extracts from rapamycin-treated H4 cells demonstrate that insulin-stimulated tyrosine phosphorylations of the 180 kDa substrate IRS-1 is unaltered by concentrations of
15 rapamycin that abolish p70 S6 kinase (data not shown).

To demonstrate that the p70 polypeptides exhibit the dominant S6 kinase activity towards 40S ribosomal subunits and to elucidate the activity of immediate upstream activators of p70 S6 kinase activity, total
20 extracts from untreated or rapamycin-treated H4 cells were resolved by Mono Q anion exchange chromatography (Fig. 5). Untreated, insulin-stimulated extracts exhibit a dominant peak of S6 activity eluting near 0.25 M NaCl, corresponding to the p70 S6 kinase. Rapamycin
25 pretreatment of H4 cells completely abrogates this peak of activity. The activity of p90 S6 kinase, which elutes from the Mono Q column in a broad peak between 0.05-0.1 M NaCl and can be detected by immunoblot (not shown), is neither enhanced nor inhibited by rapamycin (Figs. 4 and
30 5).

Insulin activates an array of proline-directed protein kinases in H4 hepatoma cells that include erk1/erk2 and a form of cdc2. These enzymes are capable of phosphorylating a putative regulatory domain at the
35 carboxy terminus of intact p70 S6 kinase. The activation

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of these enzymes can be monitored using an serine-threonine-rich synthetic peptide (SKAIPS) corresponding to these p70 regulatory sequences (Mukhopadhyay et al., J. Biol. Chem. 267:3325-3335, 1992), as well as with myelin basic protein (MBP) as substrate. At concentrations that completely inhibit activation of p70 S6 kinase, rapamycin does not alter the insulin induced phosphorylation of SKAIPS (Fig. 5) or MBP (data not shown). This result is entirely consistent with the persistence of insulin-activated p90 S6 kinase in the presence of rapamycin, inasmuch as erk/MAP kinases are the likely proximate mediator of insulin-induced p90 S6 kinase activation. Moreover, the total and Suc-1 precipitable H1 kinase activity in H4 cells is unaffected by rapamycin (data not shown).

The differential sensitivity of p70 and p90 S6 kinases to rapamycin was confirmed by examining directly the activity of the recombinant S6 kinases, expressed transiently in COS cells. The kinase activity of an epitope-tagged recombinant p70 S6 kinase was abolished completely by pretreatment of COS cells with rapamycin prior to harvest, while the activity of an epitope-tagged p90 S6 kinase and an epitope-tagged MAP kinase expressed in parallel was entirely unaffected even by 10-fold higher concentrations of rapamycin (Fig. 7A). Treatment with rapamycin did not inhibit p70 S6 kinase expression or recovery, demonstrated by an anti-p70 immunoblot of epitope-precipitated protein (data not shown). The inhibition of p70 S6 kinase activity was specific for rapamycin, as excess concentrations of FK506 failed to inhibit activity (Fig. 7B).

Transient transfection of recombinant p70 S6 kinase into COS cells results in an expression of a multiply phosphorylated polypeptide. Only a small proportion of the synthesized protein, the most highly

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phosphorylated (and thus electrophoretically slower) material is able to stimulate 40S phosphorylation. Furthermore, this recombinant p70 S6 kinase in COS cells is not regulated: neither treatment with insulin nor with phorbol ester increases its activity. Incubation of COS cells with rapamycin, however, is nevertheless able to inhibit p70 S6 kinase's ability to autophosphorylate with a concentration-dependence that precisely parallels its ability to phosphorylate 40S ribosomes (Fig. 7C). The presence of the electrophoretically retarded band in Fig. 7C indicates that a proportion of the p70 S6 kinase polypeptide is more highly phosphorylated, and while this form of p70 S6 kinase is the most active, it is also the most susceptible to inhibition by rapamycin. The disappearance of this band at 1 nM rapamycin may correlate with decreased phosphorylation of the protein itself. At higher concentrations of rapamycin, a diminution in the autophosphorylation of the lower band is also observed, suggesting that residual activity of p70 S6 kinase may be further decreased by the drug.

Concentrations of rapamycin that inhibit p70 S6 kinase activity are associated with an inhibition of the incorporation of ^3H -thymidine into serum-deprived H4 hepatoma cells in the presence or absence of insulin (Fig. 8). The complex of rapamycin bound to an FKBP has previously been shown to inhibit proliferation of cells by a lymphokine receptor signal, such as IL-2 binding to its receptor (Dumont et al., J. Immunol. 144:251-258, 1990; Bierer et al., Proc. Natl. Acad. Sci. USA 87:9231-9235, 1990). The inhibition of insulin-stimulated proliferation observed here suggests that rapamycin may have a more general effect on mitogenesis. The IC_{50} for inhibition of H4 proliferation ($\text{IC}_{50}=0.8\text{nM}$), measured at 48 hours, was somewhat lower than the IC_{50} for inhibition of p70 S6 kinase activity, measured at 60 min, in these

- 25 -

cells ($IC_{50}=5nM$). Furthermore, a component (25-50%) of proliferation remains resistant to the effects of rapamycin. The lack of a direct correlation between p70 S6 kinase activity and proliferation suggests that other biochemical signalling processes impact upon the complex function of mitogenesis. The target of rapamycin action may be upstream of both p70 S6 kinase activation and proliferation, displaying different IC_{50} s for each pathway. p70 S6 kinase may itself exert an effect on proliferation if activation of the 40S ribosomal subunit by p70 S6 kinase is a necessary step in achieving a maximal rate of proliferation. The target for rapamycin appears to be situated downstream of insulin receptor kinase and the proximal IRS-1 tyrosine phosphorylation reaction, and on a limb of signal transmission processes distinct from that mediating erk/MAP kinase activation. The rapamycin target appears to be a crucial element linking growth factor receptors to the mitogenic apparatus. This element is also likely to be a proximal upstream activator of the p70 S6 kinase, i.e., an activating p70 kinase-kinase or a regulator of such an enzyme.

Other embodiments

An assay based upon the above observations may be utilized as a means for screening for an antiproliferative or immunosuppressive agent that inhibits proliferation or immune function via a mechanism that involves the same kinase or phosphatase as that targeted by the rapamycin-FKBP complex (which may be the p70 S6 kinase, or an enzyme in the p70 S6 kinase cascade which is upstream of the p70 S6 kinase). The assay used to screen candidate compounds could use one of the cell types employed in the above-described experiments, or another cell type such as any one of the many known

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hematopoietic cell lines that are growth factor dependent, or a cell line rendered growth factor dependent by transfection with a recombinant cytokine receptor. The assay could even use yeast cells, which
5 have been shown to contain FKBP and to be sensitive to inhibition by rapamycin. As described in detail above, rapamycin-FKBP can inhibit basal proliferation of cells, so the screening assay could utilize cells which naturally exhibit a significant basal level of
10 proliferation in medium without the addition of any growth factor, cytokine, or other stimulus. Alternatively, the window of response can be maximized by using a cell line that exhibits an increased rate of proliferation in the presence of a particular mitogenic
15 stimulus. Examples of cells and appropriate mitogens include: T cell lines, B cell lines, or hematopoietic cell lines and any cytokine to which the cells are responsive, such as an interleukin (IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, or IL-11) or EPO,
20 G-CSF, M-CSF, GM-CSF or Steel factor (stem cell factor); H4 rat hepatoma cells and insulin; fibroblast cells and FGF; estrogen-responsive breast cancer cells and estrogen; or other cells which proliferate in the presence of a particular hormone or growth factor. The
25 screen could be carried out as follows: a preparation of cells is mitogen- and/or serum-starved to arrest growth temporarily, and half of the cells are exposed to the candidate antiproliferative/immunosuppressant agent, while the other half serves as control. Serum and/or an
30 appropriate mitogen are added to both cultures. Each culture is sampled at given time points, and the activity of p70 S6 kinase is measured as described above. A candidate drug which results in a significantly lower p70 S6 kinase activity relative to the control (e.g., 60% or
35 less of the control value, and preferably 20% or less) is

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classified as showing antiproliferative or immunosuppressant activity in this assay. It may then be tested in other assays, such as *in vivo* assays, in order to determine if it has the desired medical utility.

5 Alternatively, the candidate drug may be screened in a cell-free assay. In this assay, the ability of the drug to affect a particular kinase in the p70 S6 kinase cascade is tested as follows: a sample of the kinase to be tested (whether derived from natural cells or produced
10 by recombinant or synthetic means) is mixed with a peptide or protein substrate for the enzyme, ^{32}P -ATP, and the drug to be screened. Where the kinase to be tested is p70 S6 kinase itself, the substrate may be, for example, the 40S ribosomal subunit, purified S6, or an
15 appropriate fragment of S6. If the candidate drug is an analog of rapamycin which is active only when complexed with an FKBP, an appropriate FKBP must be included. A parallel incubation is performed without the drug, as a control. After a given period of incubation at 37°C ,
20 aliquots from each are taken, and the amount of ^{32}P associated with the substrate in each aliquot is determined, as a measure of the activity of the kinase in that particular aliquot. An amount of radioactivity in the treated sample higher than that in the control sample
25 is an indication that the drug activates the kinase tested, while an amount lower than in the control sample indicates that the drug inhibits the kinase tested.

As similar cell-free assay may be employed in order to screen candidate drugs for their effect on
30 phosphatases in the p70 S6 cascade. In this case, a sample of the phosphatase of interest is incubated with the candidate drug and a peptide or protein substrate which has serine and/or threonine residues already phosphorylated with ^{32}P -phosphate. The drug is absent in
35 the control. The amount of radioactivity remaining in

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the substrate after incubation is a measure of the activity of the phosphatase in this assay, and thus is a measure of the ability of the candidate drug to activate or inhibit the phosphatase.

- 5 Antiproliferative/immunosuppressant compositions which modulate the activity of some serine/threonine kinase or serine/threonine phosphatase in the p70 S6 kinase cascade, ultimately resulting in a decrease in p70 S6 kinase activity in a cell, are useful for treating
- 10 conditions characterized by unwanted proliferation of cells or unwanted immune responses. Examples of such conditions include neoplasms of any type (e.g., carcinomas, sarcomas, and leukemias); non-neoplastic conditions such as unwanted proliferation of capillaries
- 15 (as in diabetic retinopathy), of endothelial and/or smooth muscle cells in vascular walls (as in atherosclerosis), of connective tissue (as in rheumatoid arthritis), and of skin cells (as in psoriasis); autoimmune diseases; allergies; and host-vs-graft or
- 20 graft-vs-host disease resulting from transplanted cells or organs. Compositions useful in such applications include rapamycin or an analog of rapamycin, complexed with an FKBP or an analog of an FKBP; rapamycin alone or a rapamycin analog alone, if an appropriate FKBP is
- 25 present in the cells to be treated; an analog of the rapamycin/FKBP complex (made, for example, by designing a molecule which mimics the molecular structure of the critical portions of the complex); or any other composition which, when introduced into the cell to be
- 30 treated, ultimately results in a decrease in the activity of the p70 S6 kinase, by modulating the activity either of p70 S6 kinase itself, or of a kinase or phosphatase upstream of p70 S6 kinase in the p70 S6 kinase cascade. The preferred mode of treatment (whether oral or by
- 35 intravenous or other injection, by slow-release from an

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implanted source of the drug, by topical application, or by other means) and the optimal dosage regimen would be determined according to standard procedures well within the abilities of those of ordinary skill in the art.

- 5 Other embodiments are within the following claims.
What is claimed is:

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Claims

1. A method of screening for an antiproliferative or immunosuppressive agent, which method comprises contacting a eukaryotic cell with a candidate
5 antiproliferative or immunosuppressive composition; and determining the level of activity of a serine/threonine kinase or a serine/threonine phosphatase in the p70 S6 kinase cascade of said cell in the presence of said composition, wherein a level of said activity
10 that results in a lower p70 S6 kinase activity in the presence of said composition than in the absence of said composition is an indication that said composition is an antiproliferative or immunosuppressive agent.
2. The method of claim 1, wherein said
15 determination step comprises measuring the level of p70 S6 kinase activity of said cell.
3. The method of claim 1, wherein said cell is a mammalian cell.
4. The method of claim 1, wherein said cell is
20 exposed to a mitogen before or during said contacting step.
5. The method of claim 4, wherein said cell is a hematopoietic cell and said mitogen is a cytokine.
6. The method of claim 5, wherein said cell is a
25 lymphocyte and said mitogen is a lymphokine.
7. The method of claim 6, wherein a level of said activity that is lower in the presence of said compound than in the absence of said compound is an indication that said compound is an immunosuppressant.

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8. The method of claim 5, wherein said cytokine is interleukin 1 (IL-1), IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, erythropoietin (EPO), Steel factor, granulocyte colony stimulating factor (G-CSF), macrophage colony stimulating factor (M-CSF), or granulocyte/macrophage colony stimulating factor (GM-CSF).

9. The method of claim 1, wherein said serine/threonine kinase is p70 S6 kinase.

10. The method of claim 1, wherein said serine/threonine kinase is a kinase which phosphorylates and thereby activates p70 S6 kinase *in vivo*.

11. The method of claim 1, wherein said serine/threonine phosphatase is a phosphatase which dephosphorylates and thereby inactivates p70 S6 kinase *in vivo*.

12. An *in vitro* method of screening candidate antiproliferative or immunosuppressive agents, which method comprises
combining a sample comprising p70 S6 kinase, a substrate for said kinase, and a candidate composition; and

determining whether said candidate composition inhibits the biological activity of said kinase, said inhibition being an indication that said candidate composition is an antiproliferative or immunosuppressive agent.

13. The method of claim 12, wherein said candidate composition is an analog of rapamycin complexed with an FK506 binding protein (FKBP).

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14. An *in vitro* method of screening candidate antiproliferative or immunosuppressive agents, which method comprises

- providing a sample comprising p70 S6 kinase and
5 either (i) a serine/threonine kinase capable of phosphorylating and activating said p70 S6 kinase, or
(ii) a serine/threonine phosphatase capable of dephosphorylating and deactivating said p70 S6 kinase;
contacting said sample with a candidate
10 composition; and
determining whether the amount of phosphorylation of said p70 S6 kinase in the presence of said candidate composition is lower than the amount of phosphorylation of said p70 S6 kinase in the absence of said candidate
15 composition, said lower amount of said phosphorylation being an indication that said candidate composition is an antiproliferative or immunosuppressive agent.

15. A method of inhibiting cellular proliferation in response to a mitogen other than IL-2, which method
20 comprises

- providing a cell which proliferates in response to said mitogen; and
treating said cell with a composition that modulates the activity of a serine/threonine kinase or
25 serine/threonine phosphatase in the p70 S6 kinase cascade of said cell, thereby resulting in a decrease in the activity of p70 S6 kinase in said cell.

16. The method of claim 15, wherein said serine/threonine kinase is p70 S6 kinase.

30 17. The method of claim 15, wherein said serine/threonine kinase is a kinase which activates p70 S6 kinase.

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18. The method of claim 15, wherein said serine/threonine phosphatase is a phosphatase which dephosphorylates, and thereby deactivates, p70 S6 kinase.

19. The method of claim 15, wherein said
5 composition comprises rapamycin.

20. The method of claim 15, wherein said composition comprises an analog of rapamycin.

21. The method of claim 15, wherein said mitogen is IL-1, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10,
10 IL-11, G-CSF, M-CSF, GM-CSF, EPO, Steel factor, epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), or insulin.

22. An analog of rapamycin which, when introduced into a cell, modulates the activity of a serine/threonine
15 kinase or serine/threonine phosphatase of the p70 S6 kinase cascade, such that the activity of p70 S6 kinase in said cell is lower in the presence of said analog than in its absence.

23. The analog of claim 22, wherein said analog
20 forms a complex with an FKBP.

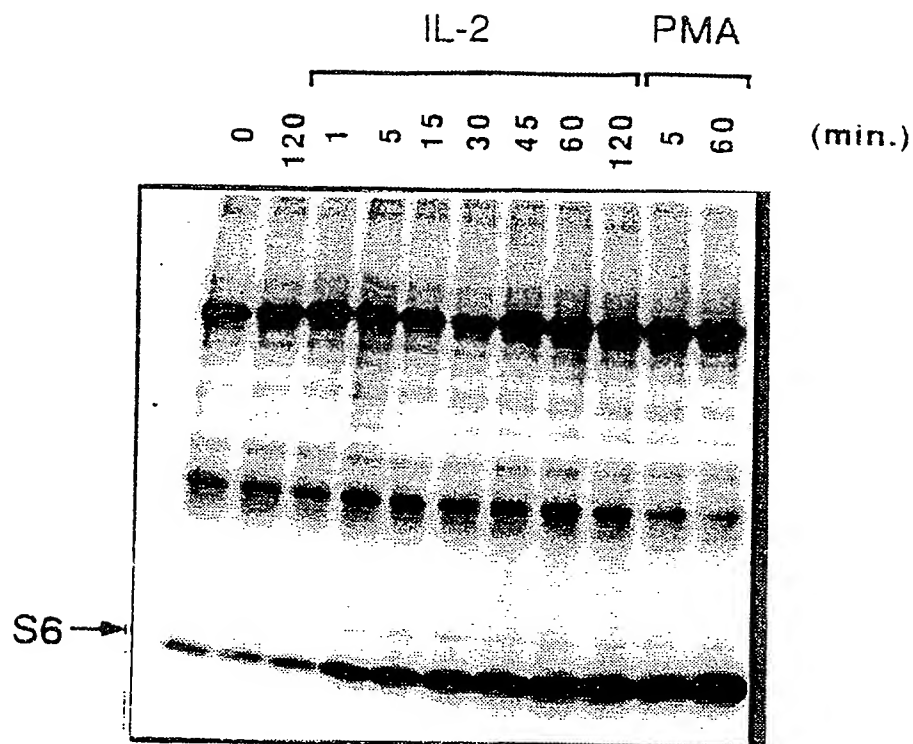


FIG. 1A

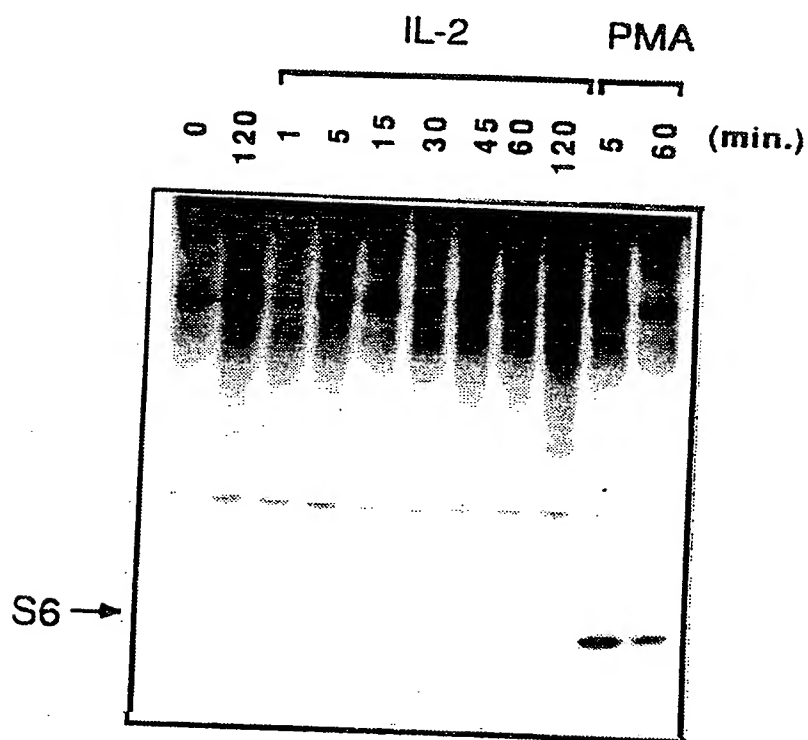


FIG. 1B

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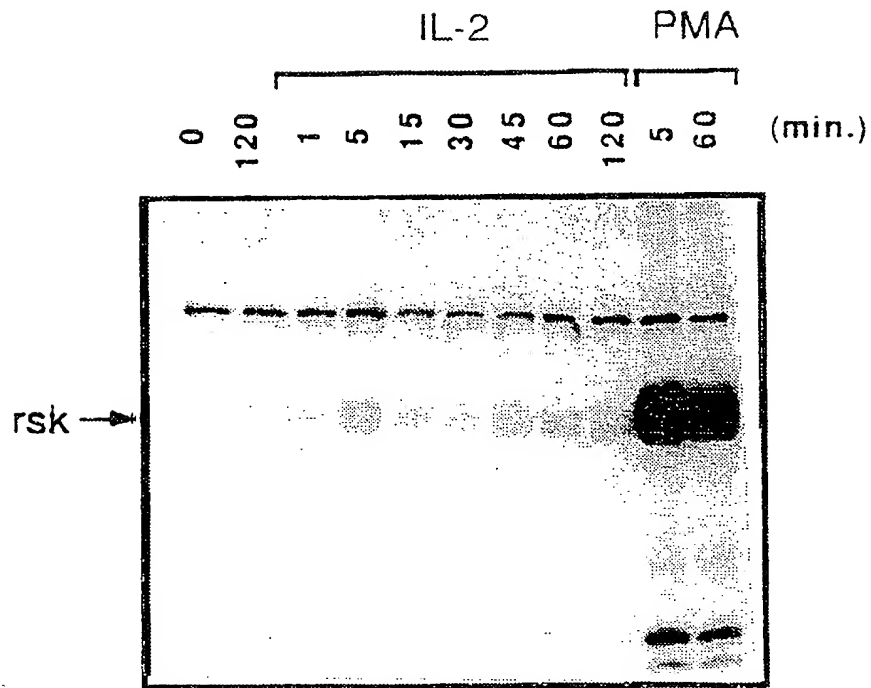


FIG. 1C

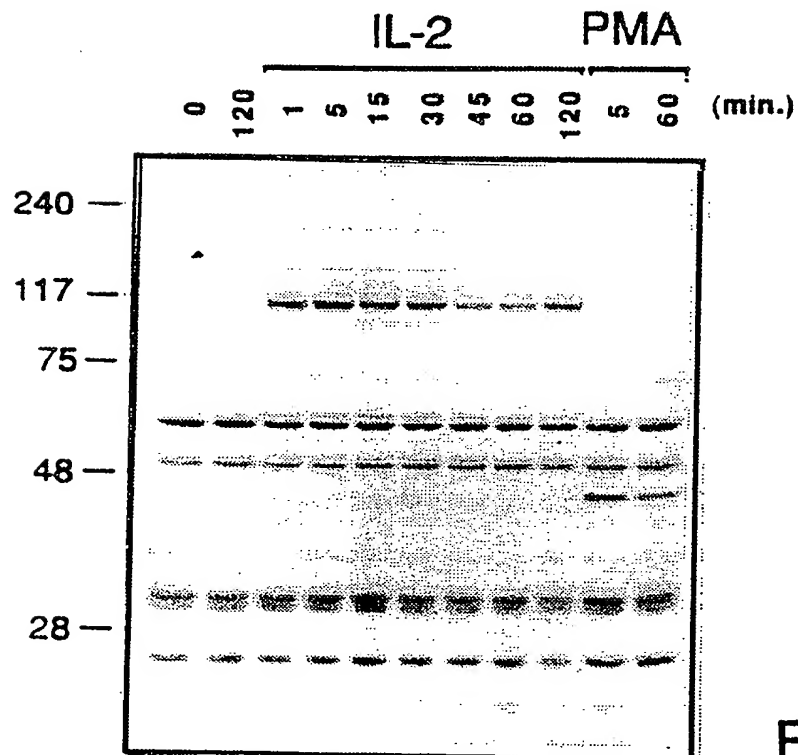


FIG. 1D

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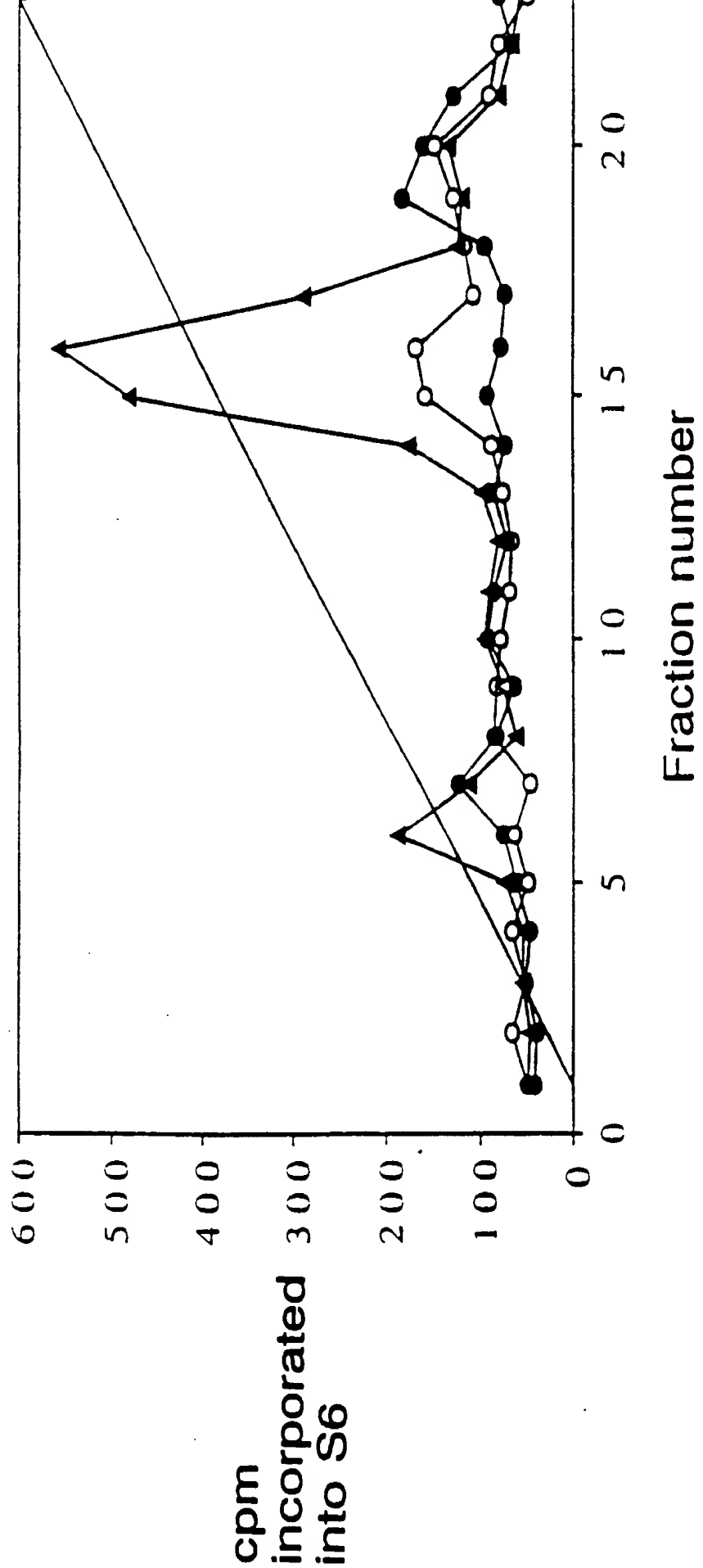


FIG. 2A

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FIG. 2B

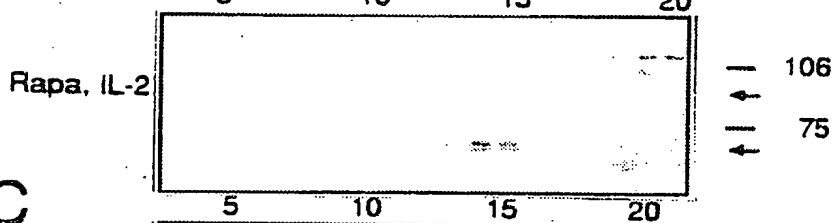
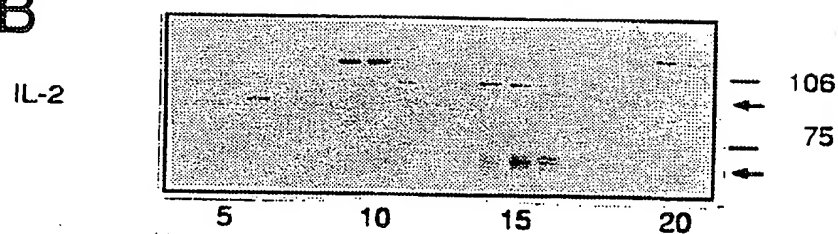
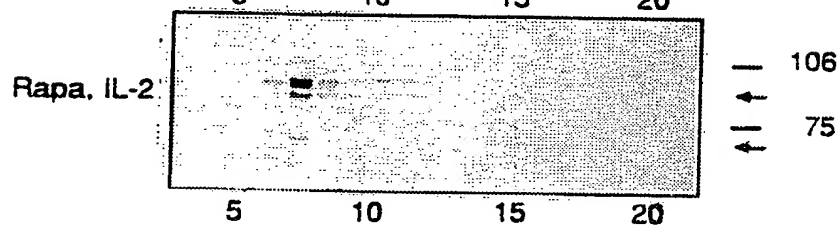
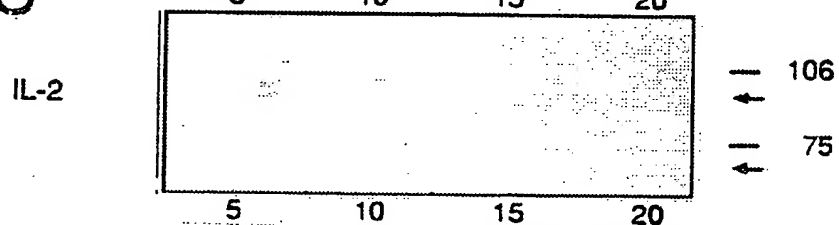


FIG. 2C



Fraction number

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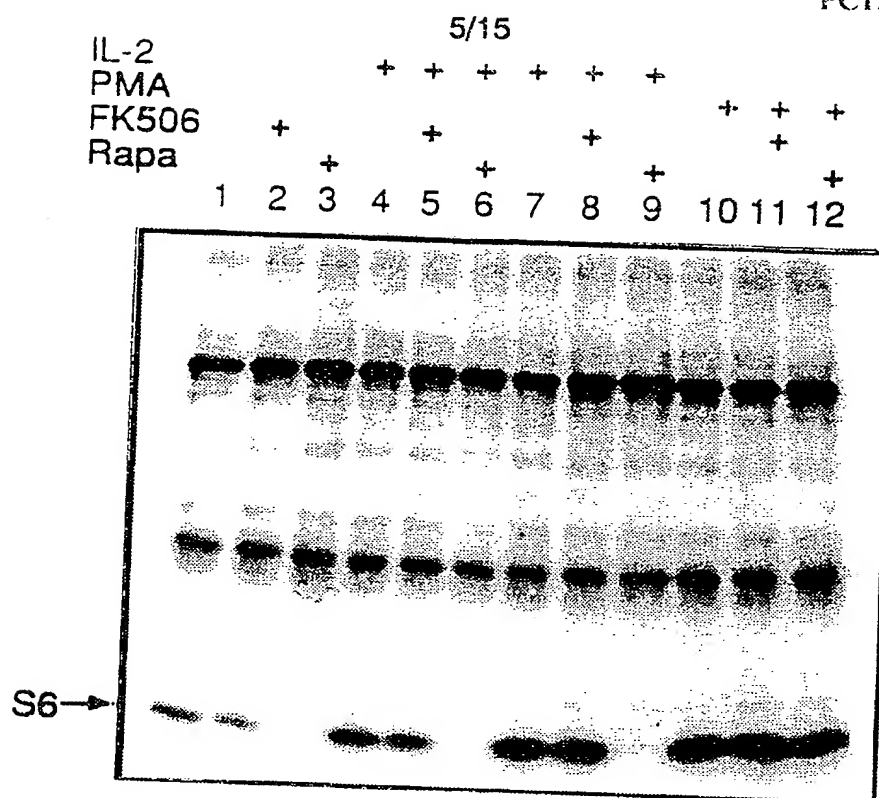


FIG. 3A

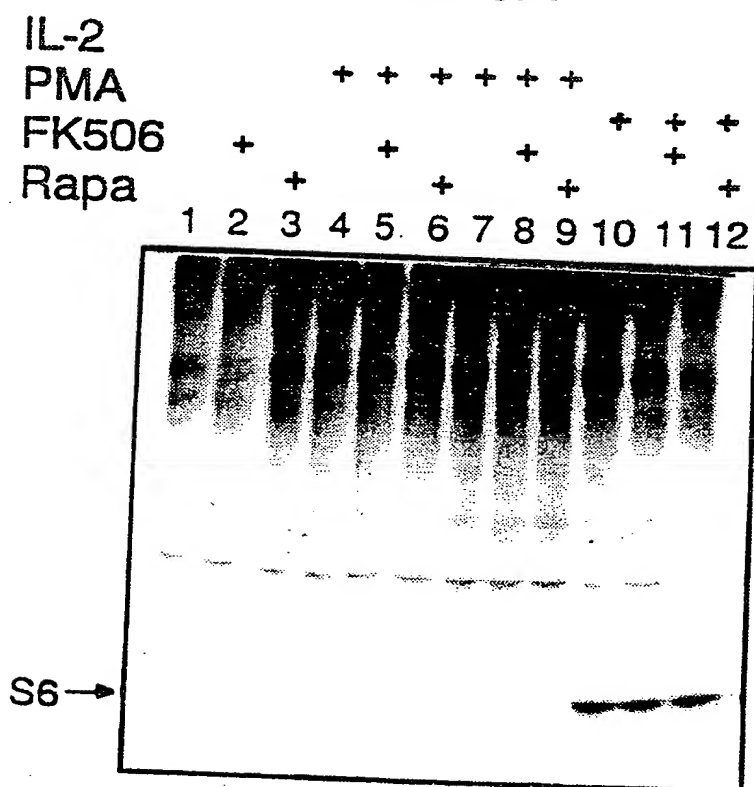


FIG. 3B

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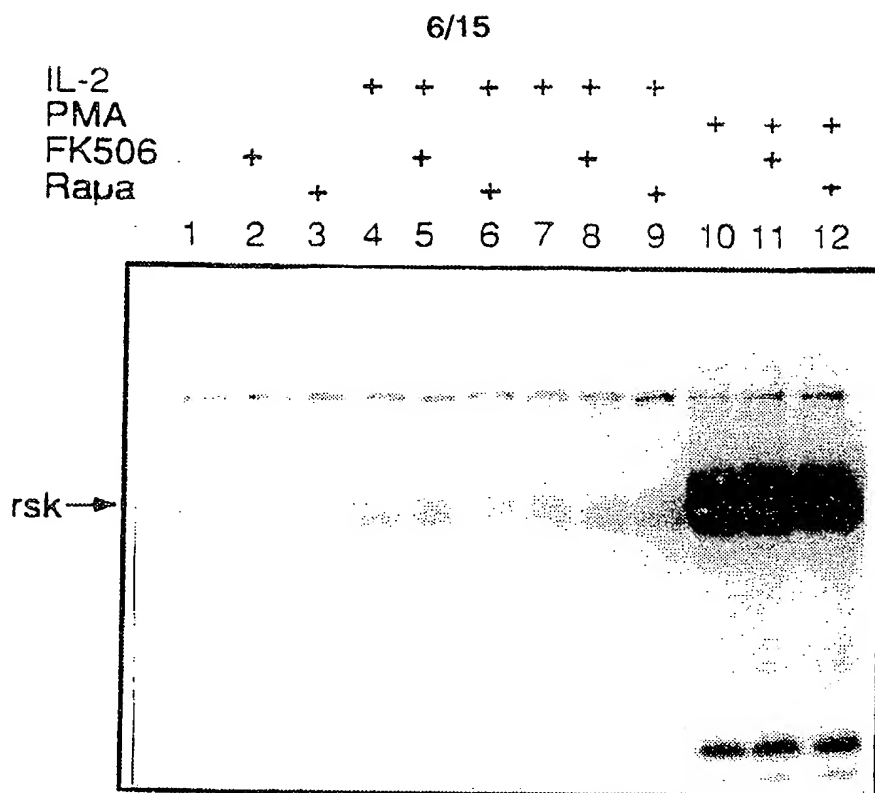


FIG. 3C

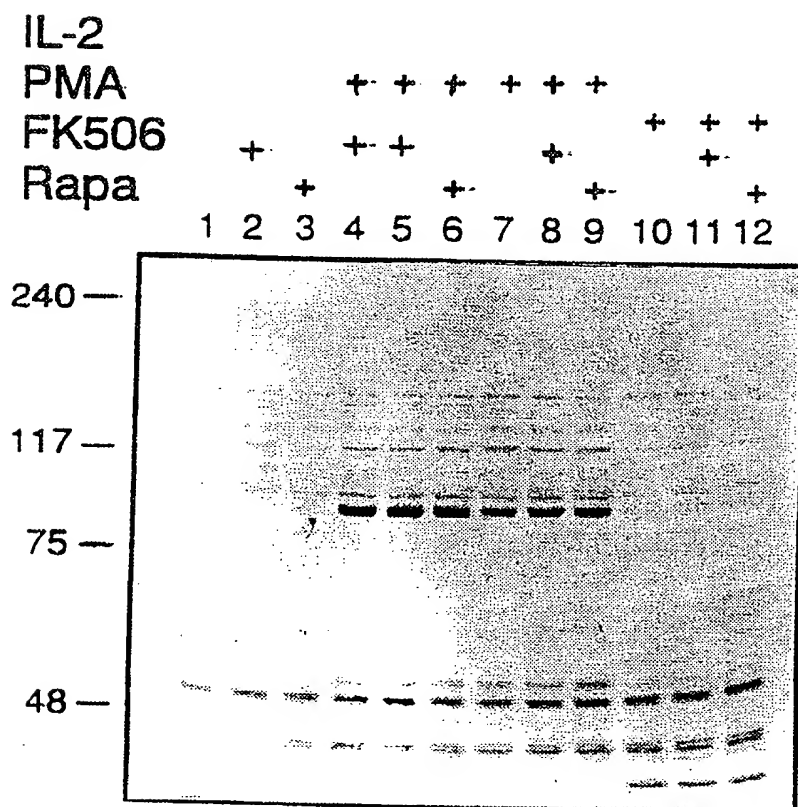


FIG. 3D

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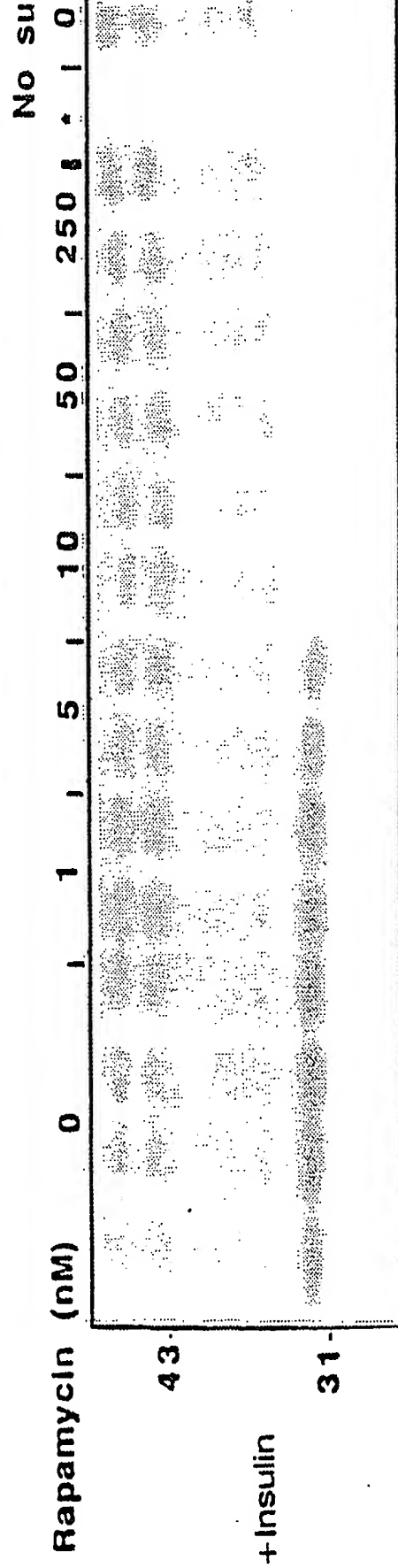
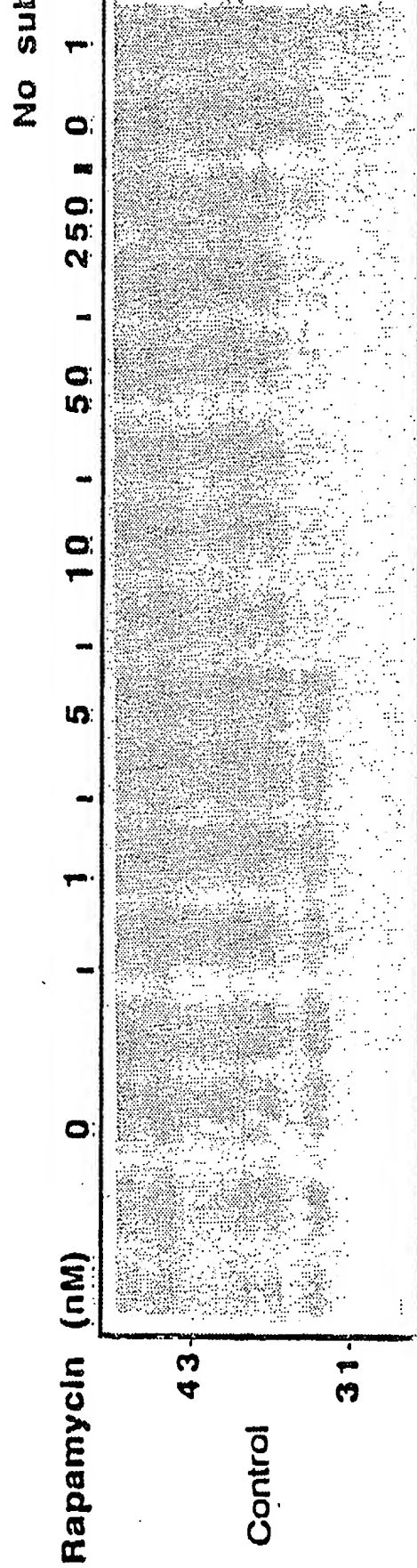


FIG. 4A

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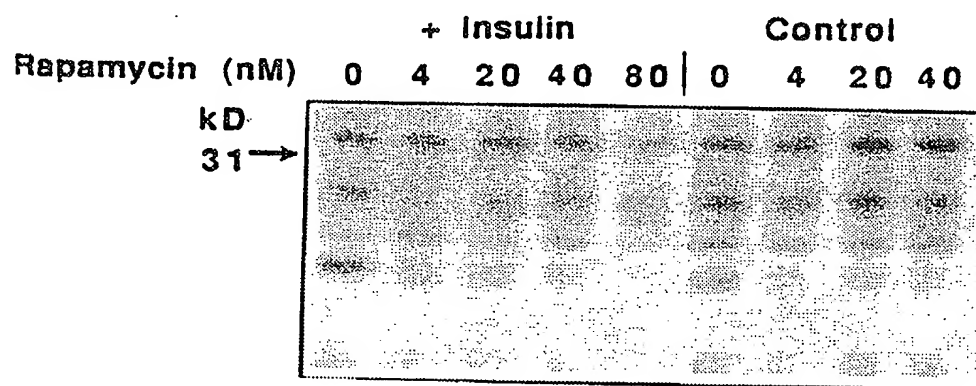


FIG. 4B

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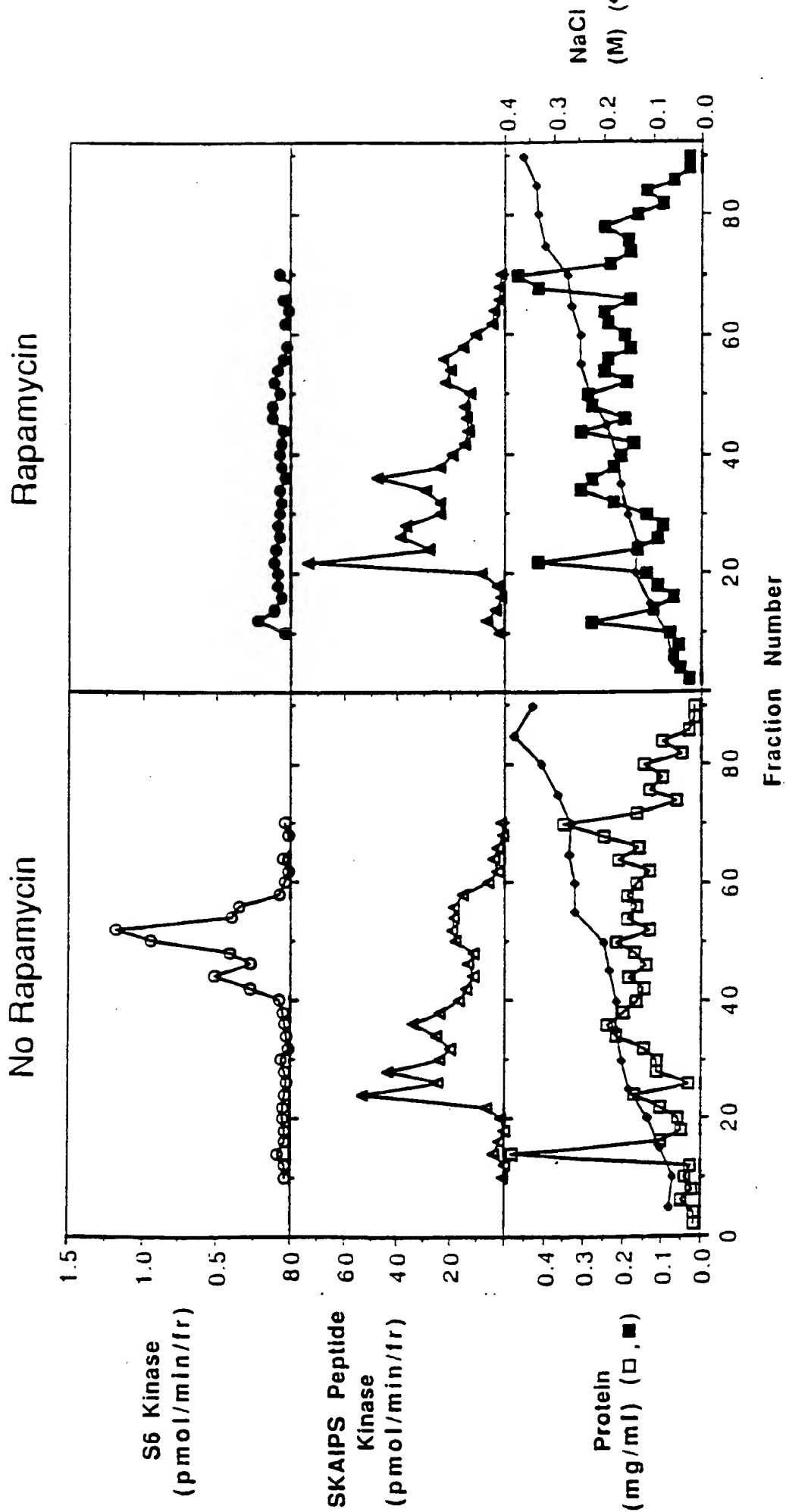


FIG. 5

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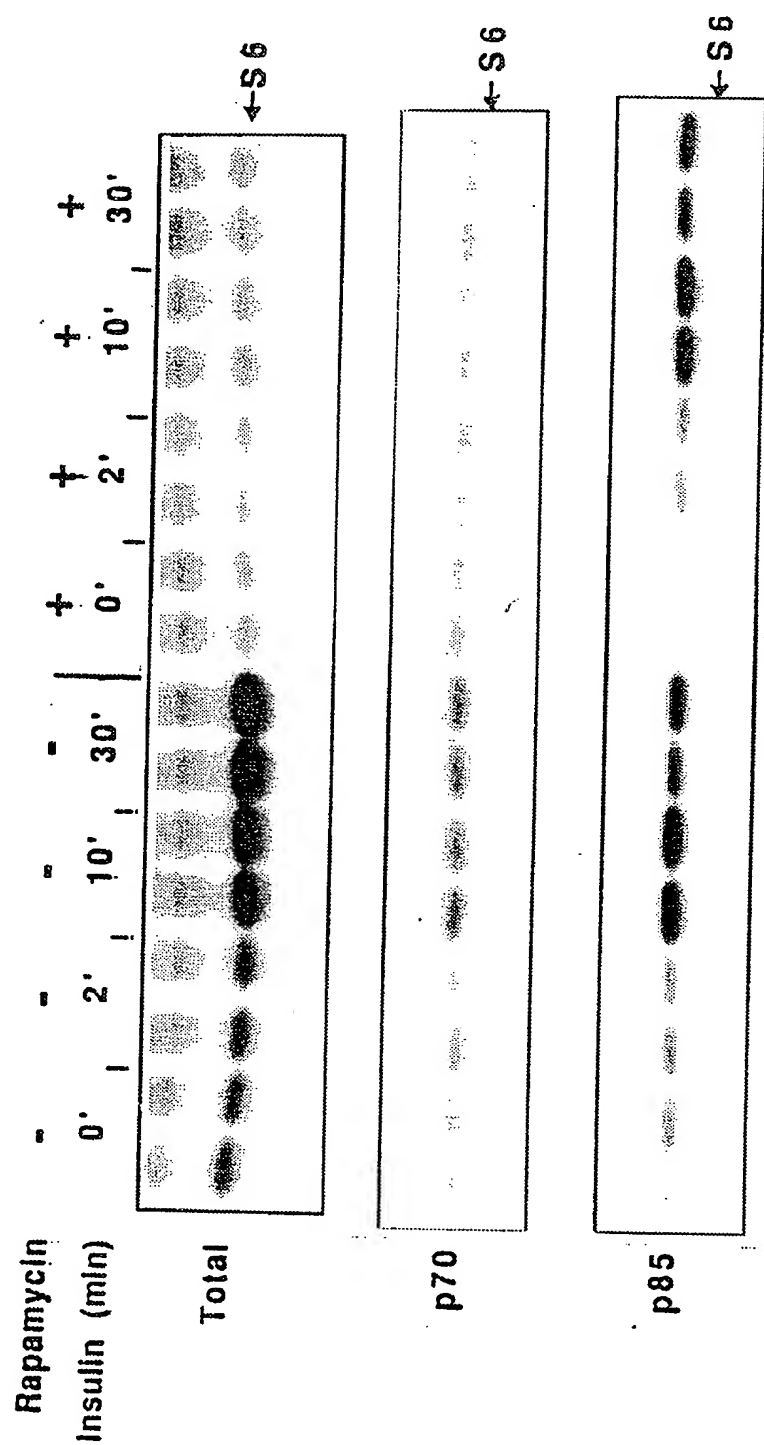


FIG. 6

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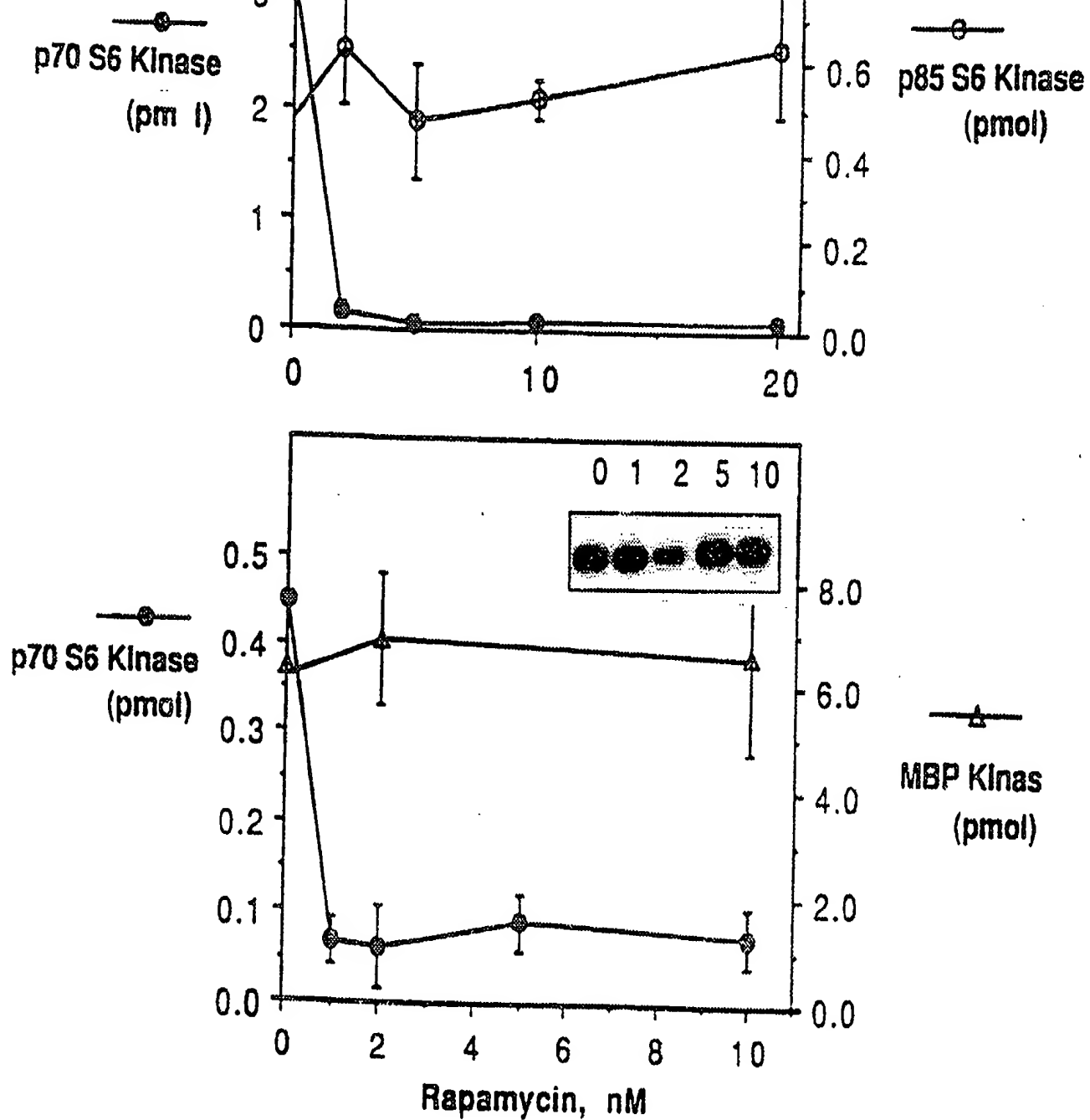


FIG. 7A

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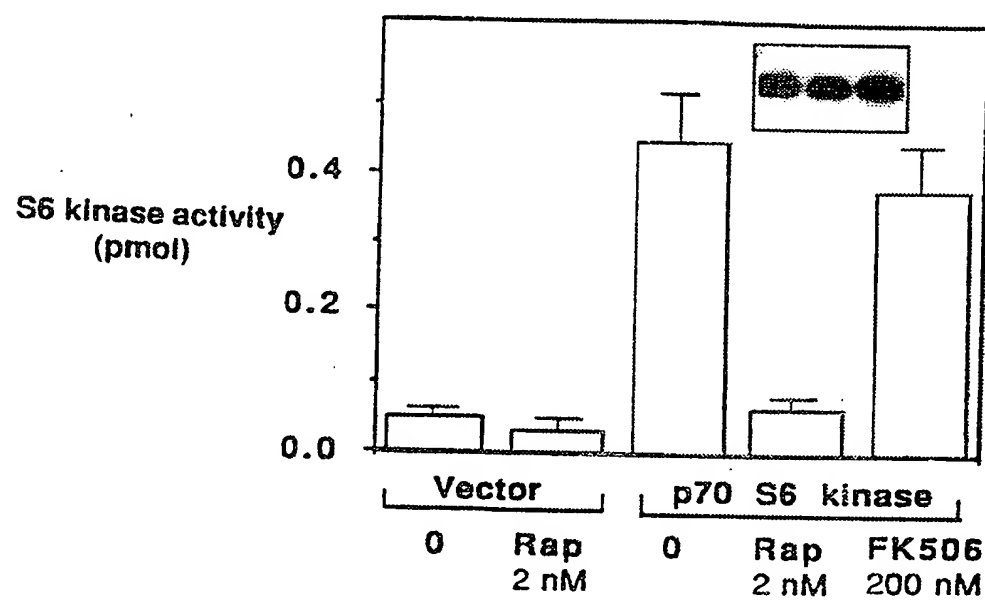


FIG. 7B

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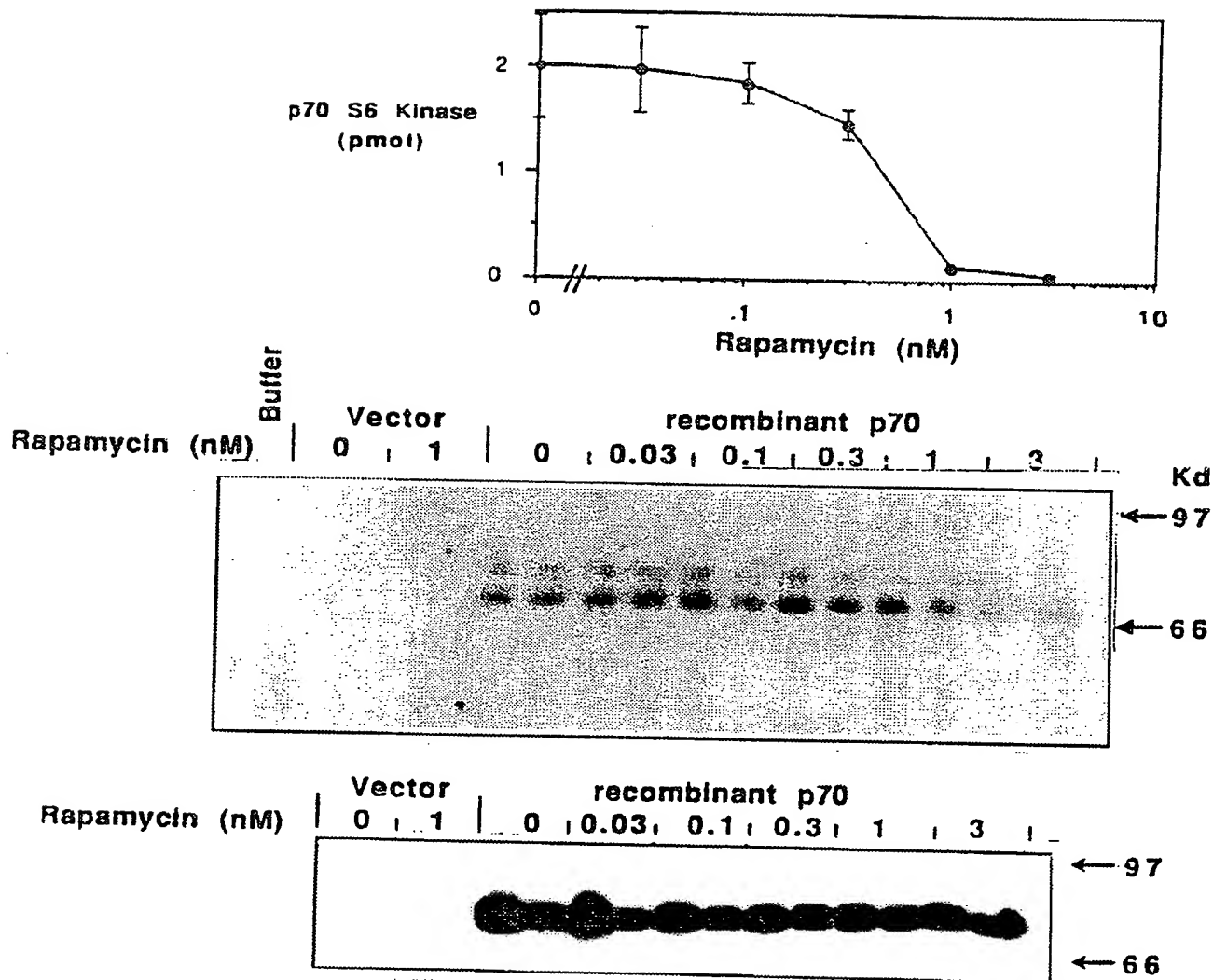
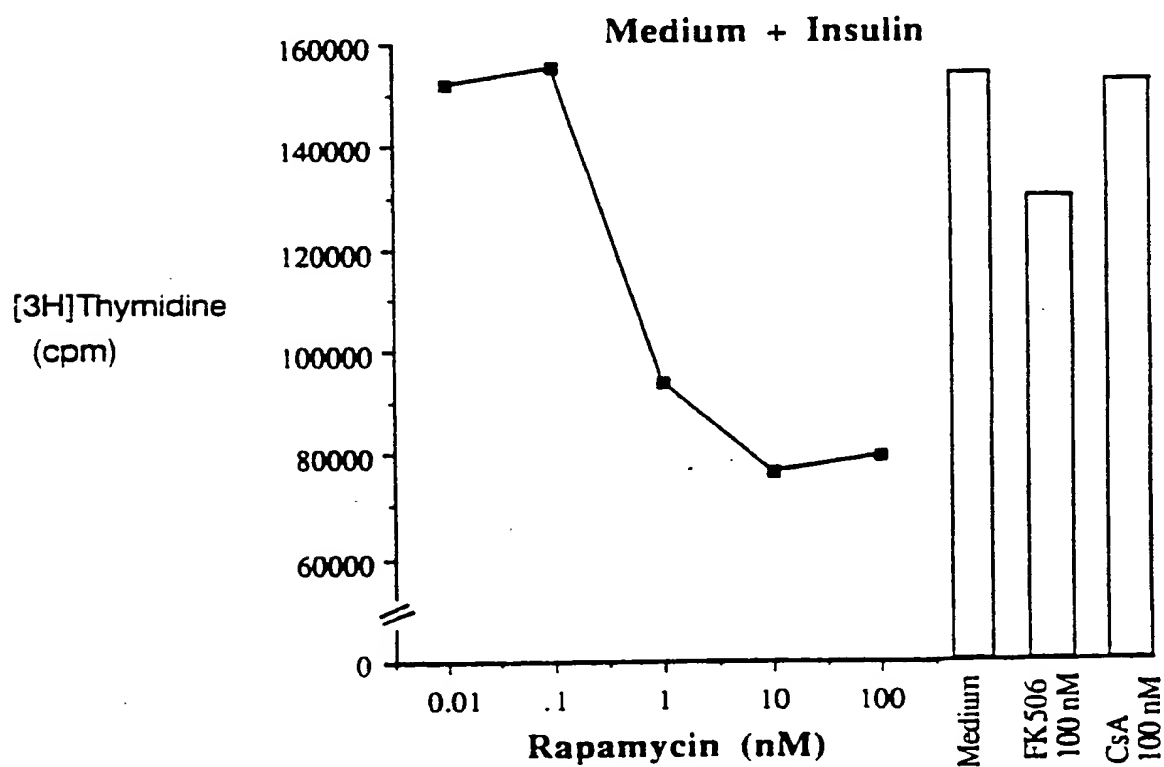
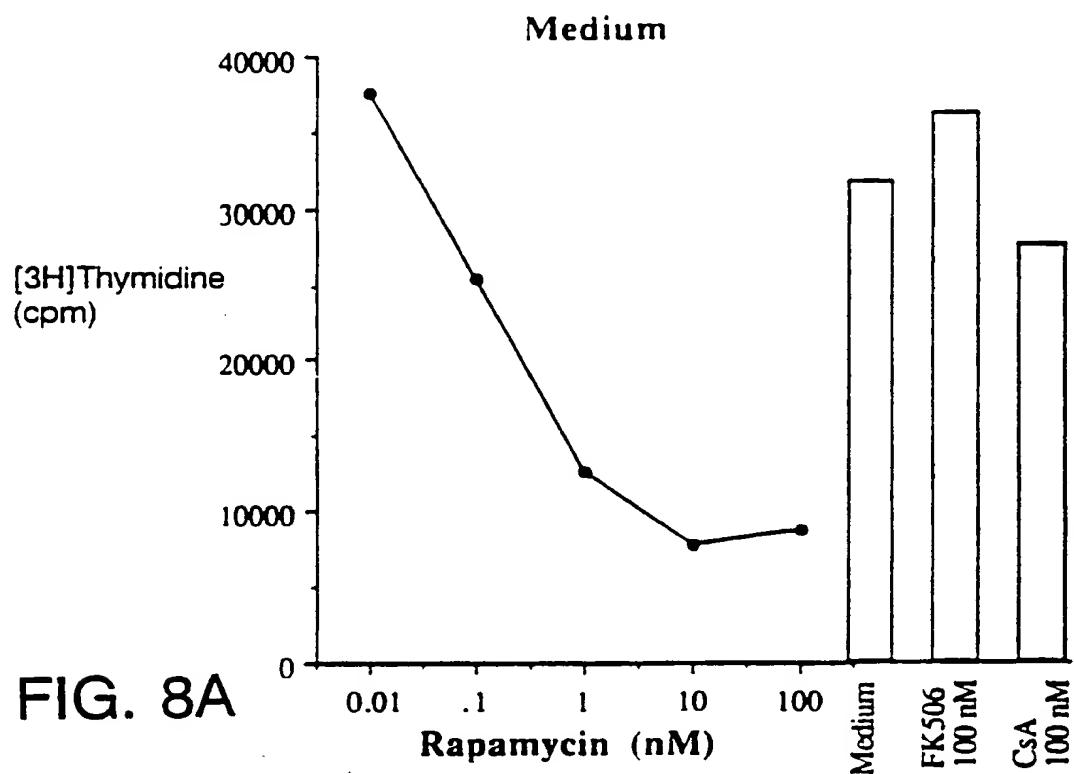


FIG. 7C

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**FIG. 8B****SUBSTITUTE SHEET**

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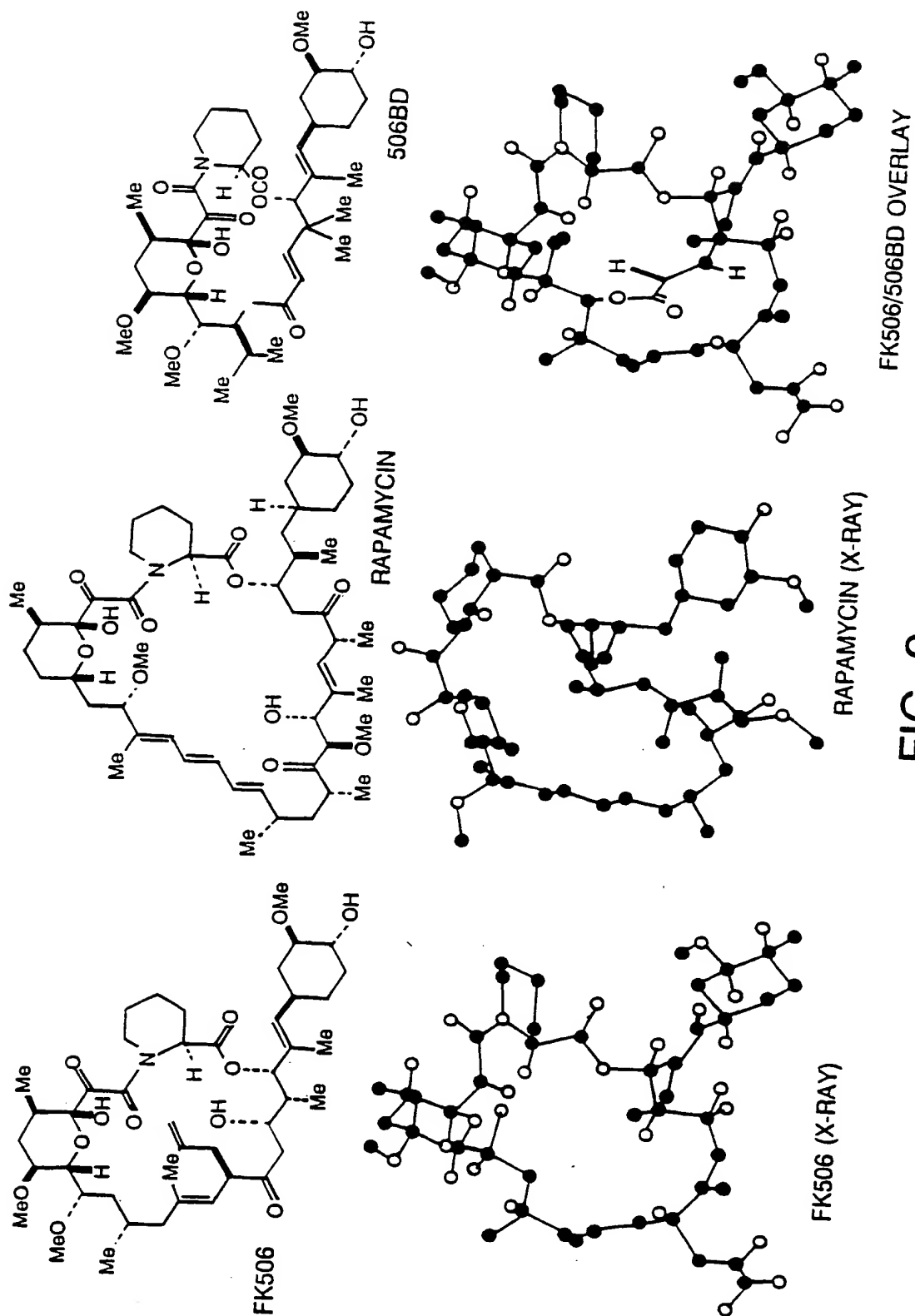


FIG. 9

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INTERNATIONAL SEARCH REPORT

I. National application No.
PCT/US93/02459

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : A61K 31/33; C12N 9/12; C12Q 1/42; G01N 33/48, 33/573; C07D 267/00

US CL : 435/7.1, 7.4, 21, 194; 436/63; 514/291; 540/455

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.1, 7.24, 7.4, 7.8, 21, 194; 436/63, 503; 514/291; 540/455, 456

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
<u>X</u> Y	JOURNAL OF IMMUNOLOGY, Vol. 144, No. 1, issued 01 January 1990, Dumont et al, "Distinct Mechanisms of Suppression of Murine T Cell Activation by the Related Macrolides FK-506 and Rapamycin", pages 251-258, see entire document, especially Abstract and pages 254-256.	<u>15-19, 21</u> 1-14, 20
<u>X</u> Y	JOURNAL OF ALLERGY AND CLINICAL IMMUNOLOGY, Vol. 89, No. 1 Part 2, issued January 1992, Hultsch et al, "Rapamycin Inhibits the Proliferation of IL-3 Dependent PT18 Mast Cells; FK506 Prevents the Effect of Rapamycin", page 238 Abstract No. 376, see entire abstract.	<u>15-19, 21</u> 1-5, 8-14, 20

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be part of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	* & *	document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means		
P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

27 MAY 1993

Date of mailing of the international search report

02 JUN 1993

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INTERNATIONAL SEARCH REPORT

In. tional application No.

PCT/US93/02459

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
<u>X</u> Y	IMMUNOLOGY, Vol. 72, issued 1991, Kay et al, "Inhibition of T and B Lymphocyte Proliferation by Rapamycin", pages 544-549, see entire document.	<u>15-19</u> 1-14, 20
Y	JOURNAL OF BIOLOGICAL CHEMISTRY, Vol. 266, No. 10, issued 05 April 1991, Erikson, "Structure, Expression, and Regulation of Protein Kinases Involved in the Phosphorylation of Ribosomal Protein S6", pages 6007-6010, see entire document, especially paragraphs bridging Col 1-2 on pages 6007 and 6008.	1-14
Y	JOURNAL OF BIOLOGICAL CHEMISTRY, Vol. 262, No. 10, issued 05 April 1987, Evans et al, "Interleukin 2 and Diacylglycerol Stimulate Phosphorylation of 40 S Ribosomal S6 Protein", pages 4624-4630, see entire document especially page 4624.	1-14
Y	CELL, Vol. 66, issued 23 August 1991, Liu et al, "Calcineurin is a Common Target of Cyclophilin-Cyclosporin A and FKBP-FK506 Complexes", pages 807-815, see especially page 807, paragraph bridging Col 1-2.	13
Y	CELL, Vol. 66, issued 06 September 1991, McKeon, "When Worlds Collide: Immunosuppressants Meet Protein Phosphatases", pages 823-826, see especially pages 823, Col 1, and 825, Col 1.	13
<u>X</u> Y	US, A, 5,093,338 (BYRNE ET AL) 03 March 1992, see Col 8-9.	<u>22-23</u> 13, 20
<u>X,P</u> Y,P	US, A, 5,102,876 (CAUFIELD) 07 April 1992, see Col 3-5.	<u>22-23</u> 13, 20
X,P	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, Vol. 186, No. 3, issued 14 August 1992, Terada et al, "Rapamycin Inhibits the Phosphorylation of p70 S6 Kinase in IL-2 and Mitogen-Activated Human T Cells", pages 1315-1321, see entire document.	1-12, 14-19
X,P	SCIENCE, Vol. 257, issued 14 August 1992, Price et al, "Rapamycin-Induced Inhibition of the 70-Kilodalton S6 Protein Kinase", pages 973-977, see entire document.	1-4, 9-12, 14-19, 21

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INTERNATIONAL SEARCH REPORT

national application No.
PCT/US93/02459

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, BIOSIS

search terms: rapamycin, analog?, antiprolif?, (prolif?(6n)inhibit?), immunosuppress?, kinase?, phosphatase?, phosphorylat?, p70

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